FUNCTIONAL STUDIES OF THE BURSICON SIGNALING PATHWAY DURING DEVELOPMENT OF *DROSOPHILA MELANOGASTER*

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The protective external cuticle of insects is not practical for accommodating growth during development. To compensate for this, the insect life cycle is punctuated by a series of molts. During the molt, a new and larger cuticle is produced underneath the old cuticle. Replacement of the smaller, old cuticle culminates with ecdysis, a stereotyped sequence of shedding behaviors. Following each ecdysis, the new cuticle must expand and harden. Studies from a variety of insect species indicate that this cuticle hardening is regulated by the neuropeptide bursicon. However, genetic evidence from the fruitfly *Drosophila melanogaster* only supports such a role for bursicon after the final ecdysis, when the adult fly emerges.

The research presented here investigates the role that bursicon has at stages of *Drosophila* development which precede adult ecdysis. In the first part of this work, I address the mechanism and timing of hormonal release from bursicon-positive motor neurons at the larval neuromuscular junction. The key findings here indicate that these peptidergic motor neuron terminals express the same core exocytotic machinery as is required for

classical neurotransmitter release, and that they secrete hormones at the neuromuscular junction in two waves of release which coincide with larval ecdysis. The second part of this work addresses the functional significance of bursicon signaling during development, by disrupting the expression of its receptor, rickets. Importantly, I found that the available *rickets* mutants are not genetic nulls as previously believed, which necessitated the use of targeted RNA interference (RNAi) to disrupt rickets expression. By eliminating *rickets* expression in different tissues by RNAi, I determined that rickets is developmentally required in the epidermis and imaginal discs for proper formation of the prepupa and to harden the cuticle before eclosion, respectively. Furthermore, I correlated the timing of lethal events resulting from *rickets* RNAi with hormonal release studies from bursicon-positive neurons. The combined results indicate that bursicon signaling is an important feature of the development of insects.

BIOGRAPHICAL SKETCH

Brandon Loveall was born atop the rolling 'West Hill' of Ithaca, NY on August 5th, 1974. A true Ithaca native, he has spent most of his adult life within 10 miles of his birthplace, slowly inching his way towards Mudd Hall, on Ithaca's opposite 'East Hill'. Brandon graduated from Bucknell University with a B.A. in biology in 1996 and quickly returned to Ithaca, where he worked a series of jobs as a lab technician. In 2004, he entered the Doctorate Program in the Department of Entomology at Cornell University and joined the lab of Dr. John Ewer. In 2006, he transferred to the Department of Neurobiology and Behavior and joined the lab of Dr. David Deitcher. In 2010, Brandon will once again leave Ithaca to move to France, where he expects to conduct post-doctoral research in Montpellier.

This dissertation is dedicated in loving memory to my mother. The strength to finish this work often came from the thought of how proud she would have been to see its completion.

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First and foremost, I thank my family for the nurture and love they have shared with me over the years. My father James, an architect, and my mother Susan, a high school French teacher, may never have understood why I wanted to study biology, but they were always supportive of my choices. On a more immediate level, my younger sister Jennifer was especially generous in sharing her house with me during my final year of graduate school. My loving fiancée Hélène has been a wonderful inspiration throughout my dissertation, always cheering my accomplishments and consoling my setbacks. Her relocation back to France was also a strong motivating factor in the completion of my dissertation.

My interest in biology developed in high school, where I had the good fortune of having a spectacular teacher, Ms. Nancy Ridenour. I am also grateful to Dr. Owen Floody of Bucknell University, my senior year research advisor who gave me my first taste of independent research. However, my research interests were poorly defined until I met Dr. Ron Hoy. I am deeply grateful to Ron for providing a unique and fertile lab environment which stimulated my interests in insect biology, and gave me the assurance that I was prepared for the rigors of graduate school. I also profusely thank Dr. John Ewer for focusing my research interests and passing on valuable knowledge and skills required for the study of insect endocrinology.

Working in Dr. David Deitcher's lab has been an immense joy and a wonderful learning experience. I could not have asked for a better advisor to help me through the process of conducting original, independent research. I cannot state everything that he has done for me to help me along this path, but I will always remember how he warmly welcomed me into his lab once John Ewer left Cornell. I have also benefited greatly from the other members of my special committee. Aside from my gratitude to Dr. Ron Hoy, I am thankful to Drs. Cole Gilbert and Ron Booker for numerous helpful scientific discussions. Cole in particular offered me a wonderful teaching assistant experience in his insect physiology course.

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CHAPTER 1
Introduction

1.1 Brief overview

For all their remarkable diversity, all insects are faced with a recurring problem during their development: the replacement of a constricting exoskeleton after periods of intermolt growth. The successful solution to this problem is to form a new, larger cuticle beneath the hardened exoskeleton. Shedding of the older exoskeleton can occur once the new cuticle is complete. A consequence of forming a larger cuticle within a smaller one is that the newly unveiled cuticle is soft and flexible; this situation is quickly rectified to provide protection against external factors such as predators and desiccation.

1.2 Description of the molting cycle

To accommodate the need for growth within a rigid external skeleton, insects must periodically replace their exoskeleton (or cuticle). The molting cycle begins with production of a larger, new cuticle directly underneath the smaller, current cuticle and reaches its apogee with the shedding of the old cuticle by way of a stereotyped behavioral routine referred to as ecdysis.

1.2.1 Events preceding ecdysis

Circulating levels of ecdysteroids (such as 20-hydroxyecdysone) are relatively low during the intermolt phase, but

dramatically rise at the end. The direct action of ecdysteroids on the epidermal cells underlying the cuticle signals a series of events which are critical to formation of the new cuticle [1, 2]. These events begin with apolysis, the separation of the epidermis from the old cuticle. Apolysis creates a cleft into which an inactive "molting gel" is secreted . Ecdysteroids also promote mitosis in epidermal cells, the secretion of a cuticulin layer, and activation of molting gel into a molting fluid. The cuticulin layer protects the underlying new cuticle from the molting fluid, which digests the endocuticle (a constituent of the old cuticle). Through these actions of ecdysteroids on the epidermis and cuticle, the stage has been set for the molting insect to shed the remaining exocuticle.

1.2.2 Shedding of the cuticle

Following the production of the new cuticle and the dissolution of the old endocuticle, the molting insect is prepared to shed the remains of the old cuticle. This is executed by way of an organized pattern of stereotyped muscular contractions, collectively referred to as ecdysis behaviors. All insects must successfully deploy these behaviors to remove the old cuticle. In spite of the variety of body forms that insects can have, the basic theme of loosening and removing the old cuticle remains the same across all ecdyses. For example, larval ecdysis in holometabolous insects such as *Drosophila* and the hornworm *Manduca sexta* appears quite trivial in comparison to molting in a hemimetabolous insect; approximately two dozen behavioral subroutines contribute to

ecdysis in crickets (cf. [3]). For this reason, the following behavioral descriptions will be based on the larval ecdyses of *Manduca* and *Drosophila*.

1.2.2.1 Pre-ecdysis movements

The behaviors used to shed the old cuticle are temporally organized into pre-ecdysis and ecdysis. Pre-ecdysis is a preparatory behavior, and it functions to loosen the old and new cuticles from each other.

Pre-ecdysis in *Manduca* is achieved by a rhythmic series of dorsal-ventral muscle contractions which synchronously occur in all abdominal segments [4, 5]. Subsequently, a series of posterior-ventral movements which alternately contract on left and right sides of the larva are incorporated into the behavioral routine [5].

Pre-ecdysis in *Drosophila* larvae is comprised of a series of anterior-posterior contractions, followed by anterior-directed rolling waves of contractions, along the dorsal surface [6]. As with *Manduca* pre-ecdysis, intervals of contractions are broken up by periods of relaxation.

1.2.2.2 Ecdysis movements

Larval ecdysis in *Manduca* [7] occurs as an anterior-directed peristaltic wave of muscular contractions. These waves initiate in the posterior abdominal segments and their movement in an anterior direction results in displacement of the old cuticle towards the posterior end. The general effect of these peristaltic waves is

to place pressure on the dorsal ecdysial suture in the thorax of the old cuticle; once this splits, the cuticle can be effectively slipped off from the body.

Muscle contractions are used to the same effect in *Drosophila* larval ecdysis: the goal is to split the old cuticle, allowing for its removal. However, there are differences in the execution. A primary difference is that unlike *Manduca* larvae, *Drosophila* larvae have no prolegs to contend with while they escape from their old cuticle. Larval ecdysis in *Drosophila* [6] commences with several forward-thrusting head movements which plant the old mouth hooks into the substrate. These movements also create an opening in the anterior old cuticle, but further cuticle detachment is required with backwards-thrusting movements. After a brief rest, ecdysis culminates with a final forward escape movement through the cuticle opening. The old cuticle is gradually sloughed off by the larva's natural locomotion.

1.3 Regulation of ecdysis behaviors

Whereas the deposition of the new cuticle is directed by circulating ecdysteroids, numerous classical endocrinology studies in Lepidoptera (primarily in *Manduca*) have determined that the subsequent movements used to loosen and remove the older cuticle are orchestrated by a suite of interacting peptide hormones (extensively reviewed in [8]). Three main peptide hormones which regulate ecdysis behaviors have been identified in Lepidoptera: eclosion hormone (EH) [9]; crustacean cardioactive peptide

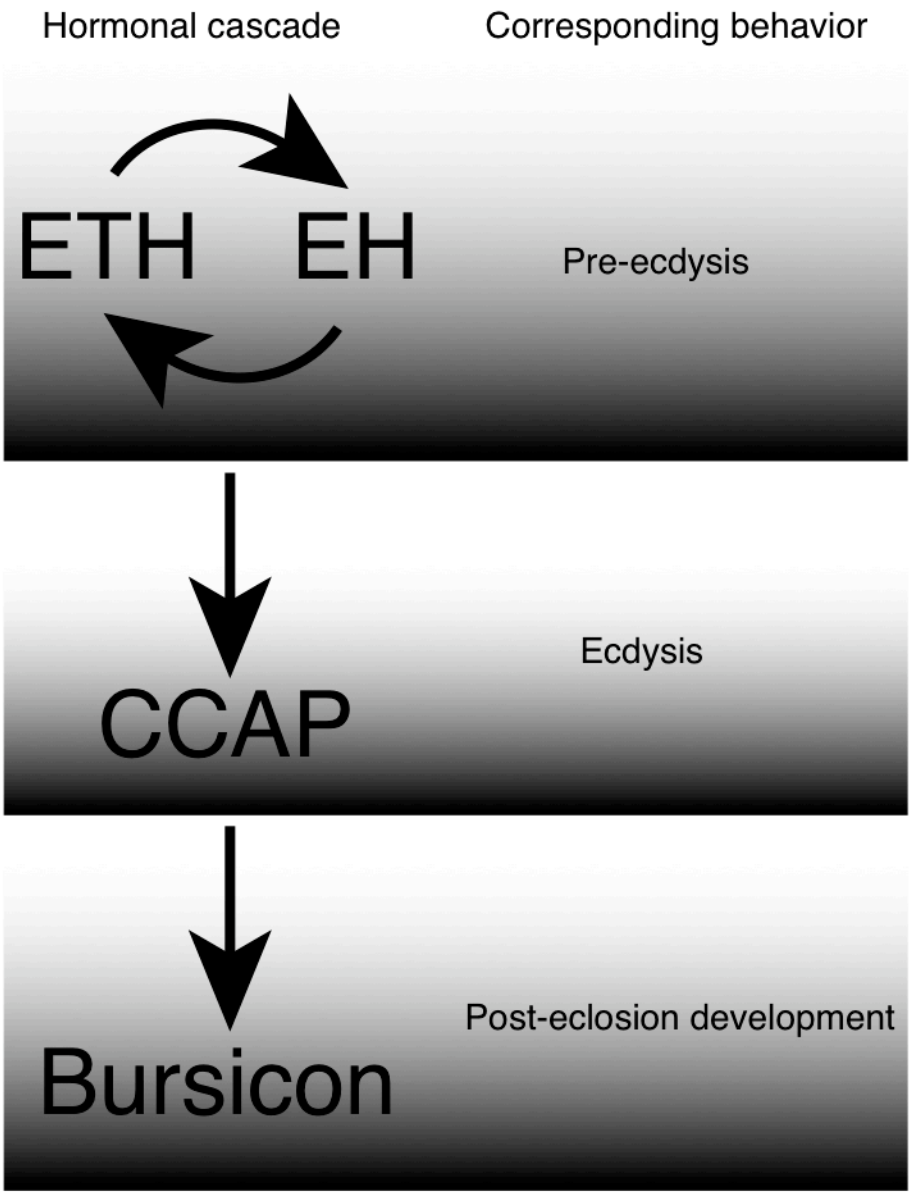
(CCAP) [10]; and ecdysis triggering hormones (ETH) [11]. Subsequent experiments with *Manduca* have lead to a model describing the temporal organization of these hormones in a pathway [12]. The canonical model of ecdysis regulation is depicted as a cascade of hormonal release (summarized in Figure 1.1), in which a positive feedback loop of ETH and EH signaling initiates pre-ecdysis [13], whereas CCAP terminates these behaviors and triggers the final ecdysis contractions required to escape from the old cuticle [14].

1.3.1 Contributions from *Drosophila*

Simply showing that a hormone is present in the right place at the right time is not proof that it functions in a given behavior. Recently, the use of *Drosophila* as a model organism for the study of ecdysis has gained popularity, due to its amenability to molecular manipulations (cf. [15]). The advantage that *Drosophila* has over other insects (such as *Manduca*) is that gene products can be functionally examined with mutants or transgenic experiments to disrupt normal protein expression.

In *Drosophila*, deletion of the *eth* gene results in lethality at the first larval ecdysis [6]. These *eth* mutants are unable to initiate organized ecdysis behaviors and consequently die at ecdysis, before the old cuticle can be removed. These results have placed ETH upstream of all other hormones which regulate ecdysis behaviors, since disruptions of EH or CCAP release do not result in (larval) lethality (cf. [15]).

Figure 1.1 The canonical model for the endocrine regulation of ecdysis behaviors. A positive feedback loop between ETH and EH signaling ensures that maximal amounts of ETH are released, committing the insect to ecdysis. ETH – EH coupled release initiates stereotyped cuticle loosening contractions (pre-ecdysis). CCAP is released downstream of ETH and EH to terminate pre-ecdysis behaviors and initiate contractions that will extricate the animal from the old cuticle (ecdysis). After adult ecdysis in *Drosophila*, bursicon has been identified as a key factor in wing expansion and cuticular tanning (post-eclosion development).


Hormonal cascade
Corresponding behavior
ETH
EH
Pre-ecdysis
CCAP
Ecdysis
Bursicon
Post-eclosion development

Whereas no *eh* or *ccap* mutants are available, various studies have used the binary GAL4/UAS transgenic system (cf. [16]) to circumvent this problem. Neurons can be ablated by expressing the cell death gene *reaper* (*rpr*) in neurons which produce the peptide hormone(s) of interest. Thus, targeted ablations of EH neurons [17, 18] (EH>*rpr*) or CCAP neurons [18, 19] (CCAP>*rpr*) have revealed that larval ecdyses can proceed in the absence of either EH or CCAP. In contrast, the behavioral program is somewhat disturbed in later ecdyses if EH or CCAP are transgenically ablated. A large proportion of CCAP>*rpr* progeny are unable to pupate, and subsequently die without successfully executing pupal ecdysis [19]. CCAP>*rpr* flies which survive through eclosion appear strikingly different from wild type flies, in that the ablation of CCAP neurons prevents wing expansion following eclosion [19]. Similarly, a fraction of EH>*rpr* flies survive without any major defects in ecydsis, but are unable to expand their wings [17] after eclosion. Wing expansion is discussed below in section 1.5.

Curiously, only the genetic deletion of ETH is capable of completely inhibiting larval ecdysis, whereas the ablation of EH or CCAP neurons only affects later developmental events. This suggests that larval ecdysis may be more complicated than the three-hormone pathway depicted in Figure 1.1. Ablation of both EH and CCAP neurons [18] (EH+CCAP>*rpr*) affects larval ecdysis much more severely than loss of either EH or CCAP neurons alone, resulting in greater lethality due to unsuccessful ecdysis.

The combined results of EH and CCAP ablations implicates redundancy within the hormonal cascade, as well as additional interactions between these hormones (cf. [18].

Identification of the ETH receptor (ETHR) in *Drosophila* [20] has been instrumental in confirming the temporal sequence of events during (pupal) ecdysis [21]. The observation that ecdysis cannot proceed without ETH [6] places ETH at the top of the peptide hormone cascade which regulates ecdysis. If ETH signaling ultimately causes the release of other hormones in the cascade, this relationship can be exploited to investigate how release of the various 'downstream' hormones is assembled to elicit ecdysis behaviors. Indeed, ETHR-expressing peptidergic neurons respond to *in vitro* ETH stimulation (which prematurely initiates ecdysis) with increased Ca^{2+} levels [21]. Neurons which release EH and CCAP respond to ETH with different time courses for Ca^{2+} activity (measured by transgenic expression of a Ca^{2+} sensitive GFP in these neurons), in a manner consistent with their roles in triggering pupal pre-ecdysis and ecdysis behaviors [21].

These results support a model where ETH orchestrates a complex sequence of peptidergic neuron activation, with the caveat that ecdysis was artificially induced in pupae by challenging them with injected ETH. This approach is unlikely to identify peptide hormones which act upstream or parallel to ETH activation. For example, in *Manduca* the direct application of the neuropeptide corazonin to isolated Inka glands (which produce ETH) causes the release of ETH [22], although such a relationship between

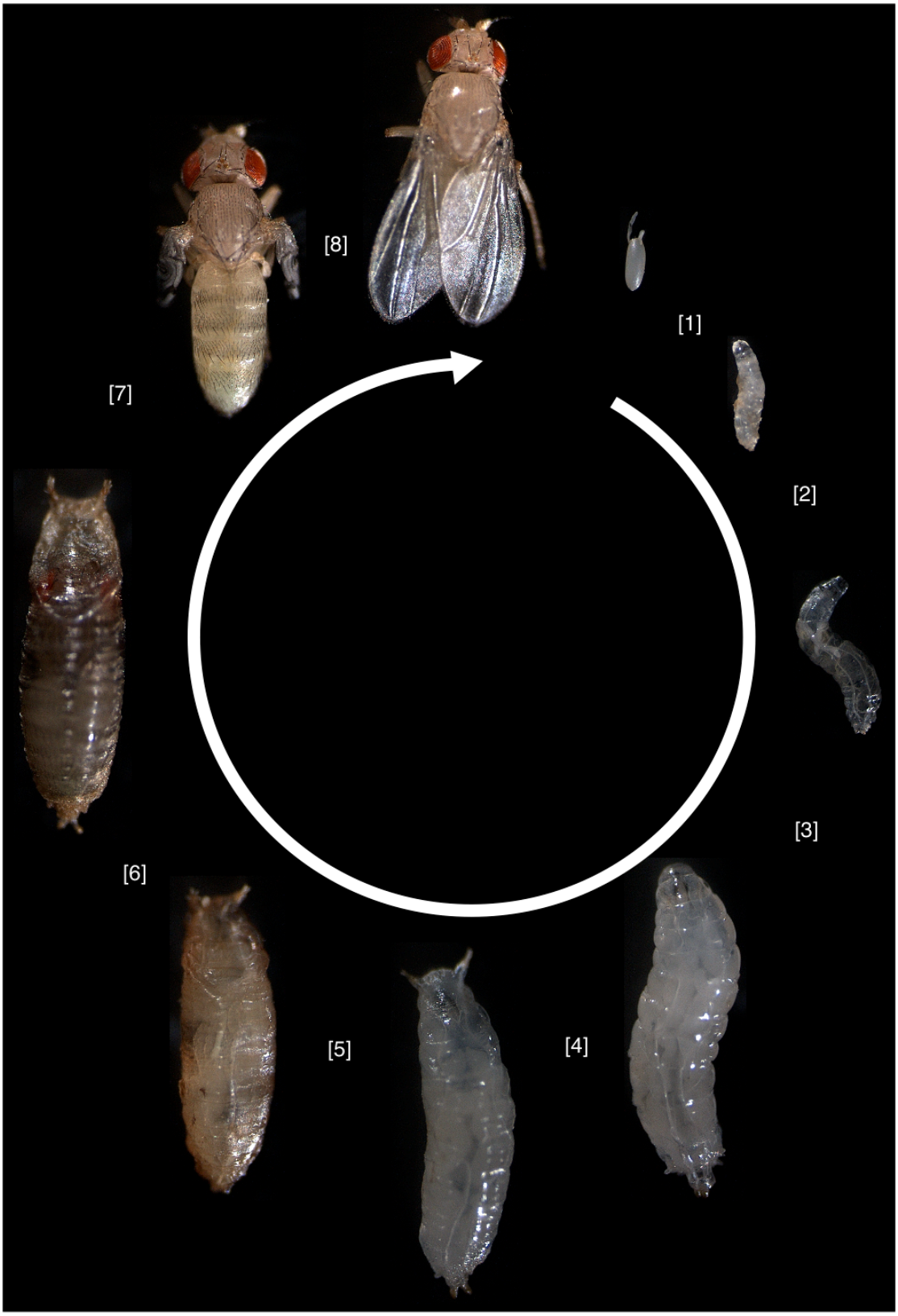
corazonin and ETH has not been identified in *Drosophila* (cf. [15]). However, in *Drosophila* ablation of both EH and CCAP neurons (EH+CCAP>*rpr*) results in a delayed initiation of pre-ecdysis behaviors (relative to wild type animals) [18]. This delay suggests that EH and CCAP neurons may also somehow function upstream of ETH signaling.

1.4 Developmental transitions in which ecdysis occurs in *Drosophila*

The life cycle of *Drosophila* contains many transitions, several of which require ecdysis behaviors as described above in sections 1.2.2 and 1.3. These transitions are summarized in Figure 1.2. The hormonal cascade which initiates ecdysis behaviors is similar at each molt, however there are key differences in the displayed behaviors at the different transitions.

Hatching of the embryo does not constitute an ecdysis, and accordingly there has been no emphasis on determining the roles of ETH, EH or CCAP at this transition. *Drosophila* larvae molt twice, for a total of three larval instars. These ecdyses resemble the behavioral descriptions in section 1.2.2. Two important morphological markers have been described to facilitate the recognition of *Drosophila* larvae approaching pre-ecdysis [6], which are based on the duplication of the new cuticle. Depending on whether it is the 1st or 2nd larval ecdysis, the appearance of a new set of mouth hooks on top of the old mouth hooks (or 'double mouth hooks') can precede ecdysis behaviors by 30 minutes to 90

Figure 1.2 Developmental transitions in *Drosophila melanogaster*. [1] Hatching of the 1st instar larva from the embryo is not typically considered as an ecdysis. Ecdysis (as portrayed in Figure 1.1) occurs at the transition from a 1st instar larva into a 2nd instar larva [2], and again at the transition from a 2nd instar larva into a 3rd instar larva [3]. Pupariation, the transition of a 3rd instar larva into a prepupa [4], is not an ecdysis but rather the formation of a puparium from the 3rd instar cuticle. As the puparium tans [5], the developing prepupa undergoes pupal ecdysis (pupation) [6] to form a pharate adult. After the final ecdysis (eclosion) [7], the newly hatched adult fly has a soft cuticle and unexpanded wings. Shortly after hatching, the wings expand and the cuticle hardens [8] in a process regulated by the hormone bursicon.


[1]
[2]
[3]
[4]
[5]
[6]
[7]
[8]

minutes. Following this, the appearance of a complete set of mouthparts (or 'double vertical plates') , which includes the cephalopharyngeal skeleton, has been correlated with the secretion of ETH.

Almost all holometabolous insects molt from the final larval instar into a pupa, in an ecdysis that is also referred to as pupation (or pupal ecdysis). A notable exception are the cyclorraphous Dipterans (or Muscomorpha) such as *Drosophila*, houseflies, and blowflies. These flies do not pupate at the end of their larval stage, but rather they form a hardened puparium (or pupal case) from the 3rd instar cuticle [8]. Unlike the tanning that occurs with maturation of the adult cuticle, tanning of the puparium is not believed to be mediated through bursicon activity but instead by a 'puparium tanning factor' (PTF), identified in the flesh fly *Sarcophaga bullata* [23-25]. Although a likely ortholog exists in *Drosophila* [25], this model organism has not been used to examine the role of a PTF at pupariation. It is within this puparium that the pupa forms, performs pupal ecdysis, and undergoes metamorphosis [8]. Pupal ecdysis behaviors [21] differ considerably from the larval ecdyses. The key difference with pupal ecdysis is that the old cuticle (the puparium) is not removed. Instead, ecdysis behaviors at pupation are used to evert the head, which has developed internally within the thorax [21].

The final developmental transition to employ ecdysis is hatching of the adult (eclosion). The significant behaviors used at *Drosophila* eclosion [26] include inflation of the ptilinum (an

expandable patch of cuticle on the head), and stylized contractions of thoracic and abdominal muscles. Ptilinum expansion opens the anterior surface of the puparium (at the operculum), through which the fly can extricate itself with the subsequent muscle contractions.

1.5 Post-eclosion development

Drosophila attain their mature form shortly after eclosion, once the wings expand and the new cuticle tans (undergoes sclerotization and melanization). Several lines of evidence support a role for the heterodimeric neuropeptide bursicon, through its receptor rickets, in regulating these aspects of post-eclosion development. Genetic mutations in either rickets (*rk*) [27] or the bursicon subunit (*burs*) [28] prevent wing expansion and delay cuticular tanning.

A subset of CCAP-expressing neurons co-expresses bursicon; at eclosion, the release of bursicon is regulated by bursicon-negative CCAP cells [29]. The bursicon-positive CCAP neurons can be subdivided into two regions: excitability of bursicon neurons in the abdominal ganglion is required to regulates cuticular tanning, whereas excitation of bursicon neurons in the subesophageal ganglion is necessary to mediate the wing expansion behavioral program [30]. Peripherally, bursicon mediates the tanning of the new cuticle likely through rickets activation at the epidermis (cf. [31]). Dopamine is a critical molecule for tanning the new cuticle, and the synthesis of its precursor dopa from tyrosine requires the enzyme tyrosine

hydroxylase (TH), encoded by the *Drosophila* gene *pale* [32]. Both *burs* and *rk* mutants are unable to phosphorylate TH [33], although TH phosphorylation can be rescued in these mutants if they are injected with a membrane-permeable variant of cAMP [33]. These results provide a link between bursicon signaling and post-eclosion cuticle tanning, since the rickets receptor uses cAMP as its second messenger [34, 35].

1.6 Perspectives

In this chapter, the insect molting cycle and its hormonal control were reviewed. As described in section 1.5, the hormone bursicon (through its receptor, rickets) is a key factor in tanning the new cuticle. However, direct and incontrovertible genetic evidence for the temporal activity of bursicon/rickets has only been demonstrated in post-eclosion *Drosophila*. In chapter 2 of this dissertation, the mechanism and timing of bursicon release is examined at the 2nd larval ecdysis, from specialized neuronal terminals at the neuromuscular junction. This theme is extended in chapter 3, which examines potential bursicon release during pupal development. Chapter 3 also considers functional aspects of bursicon signaling prior to eclosion, by manipulating expression of the *rickets* gene in different tissues. A summary and general discussion of the results are to be found in chapter 4.

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CHAPTER 2

Secretory protein distribution and novel ecdysis-related vesicle release from type III boutons at the *Drosophila melanogaster* larval NMJ

2.1 Abstract

At the *Drosophila* larval neuromuscular junction (NMJ), a unique class of 'type III' boutons is specialized in the storage and release of dense core vesicles (DCV) packaged with peptide hormones. These boutons represent the terminals of motor neuron axons which project from select 'crustacean cardioactive peptide' (CCAP) neurons in the ventral ganglion. Based on the identities of neuropeptides present in these neurons, it is likely that the type III boutons serve a neuropeptide release function at ecdysis. However, both the mechanism and functional significance of release from these boutons has been overlooked in development. To address the issue of type III bouton secretion in larvae, we have established which secretory proteins colocalize with the hormone bursicon. In type III boutons or their motor neuron axons we found neuronal Synaptobrevin (N-SYB), Cysteine String Protein (CSP), Syntaxin 1A (Syx1A), and intriguingly a combination of SNAP-24 and SNAP-25. Furthermore, the expression of Syx1A RNAi in CCAP neurons (a subset of which project and terminate as type III boutons at the NMJ) phenocopies genetic mutants in the bursicon

signaling pathway. Additionally, we measured vesicle release from type III boutons by measuring fluorescence intensity changes with the targeted GFP-tagged neuropeptide, ANF-EMD. We observed two phases of vesicle release from type III boutons during a narrow developmental window. The first phase was before the initiation of ecdysis while the second phase extends for several hours beyond the completion of this vital insect behavior. Our analysis of the secretory proteins present in type III boutons indicates that the secretory machinery required for DCV release is similar to that necessary for the release of "classic" neurotransmitters. Of particular interest is the dual expression of both SNAP-24 and SNAP-25, recalling a previously described redundancy for these two proteins at the *Drosophila* larval NMJ. Significantly, the type III boutons release their DCV at two phases, which precede and follow the 2nd larval ecdysis. This firmly establishes a role for the peptide hormones released from these boutons (such as bursicon or CCAP) as relevant signals to the periphery during a crucial stage of development.

2.2 Introduction

Neuropeptides play a fundamental role in the regulation of behavior, development, and physiology in invertebrates and vertebrates alike [1, 2]. Characteristically, these hormones perform their peripheral functions at a distance from their sites of secretion in the CNS. In *Drosophila*, a typical mode of action is the central

release of neuropeptides into the hemolymph by the *corpora cardiaca* [3]. Intriguingly, neuropeptides are also released peripherally from motor neuron terminals at the NMJ. These putatively "neurohemal" release sites (cf. [4, 5]) are the type III boutons, located alongside the type I and type II boutons of muscles 12 (NMJ 12) (and occasionally 13) at body segments T3-A4.

Whereas the excitatory transmitter glutamate is present at type I, and to a lesser extent, type II boutons (along with octopamine) [6, 7], the ultrastructure of the type III boutons indicates a specialization for peptidergic release [6]. This morphological evidence is supported by a number of studies that have established immunoreactivity to several different neuropeptides in the type III boutons, including an insulin-like peptide [8], CCAP [9], leucokinin-like immunoreactivity [10], and myoinhibiting peptides (MIP) [5]. In contrast to our understanding of the morphology of type III boutons and their content, both their mechanism of vesicular release and the significance of their release at the NMJ remain elusive.

Are the proteins believed to be essential to neurotransmitter exocytosis also expressed in type III boutons? The docking and release of packaged transmitters is facilitated by a wealth of secretory proteins, including the SNARE proteins syntaxin, SNAP-25, and synaptobrevin (reviewed in [11]). Briefly, the SNARE hypothesis proposes that protein-protein interactions between the vesicle SNARE synaptobrevin and the target membrane SNAREs

syntaxin and SNAP-25 mediate the fusion of vesicles with the plasma membrane. In *Drosophila*, the neuronal SNAREs are encoded by *n-syb*, *Syx1A*, and *Snap-24* and *Snap-25*, Another vesicular membrane protein CSP appears to have an important role in the process, although its putative role as a protein chaperone has not been clarified [11]. The importance of these proteins to synaptic vesicle release of neurotransmitters has been well studied in *Drosophila*, *C. elegans*, and mice [12]. However, little has been done to demonstrate the role of SNARE proteins in neuropeptide secretion in a genetic model system.

Could the role of neuropeptides released from type III boutons be to modulate behaviors associated with larval or pupal ecdyses? The cuticle-shedding behaviors collectively known as ecdysis are orchestrated by a suite of interacting peptide hormones including ecdysis-triggering hormone (ETH), eclosion hormone (EH) and CCAP (reviewed extensively in [13-15]). This model proposes that coupled release of ETH and EH initiates the preparatory behaviors of ecdysis; at least in *Manduca sexta* larvae, CCAP terminates these early behaviors and also serves to trigger the final bouts of shedding the exuvia [16]. However the requirement for CCAP at *Drosophila* larval ecdysis is not as clear [17, 18]. In the case of adult eclosion, a fourth secreted neuropeptide, bursicon, is required for both cuticle tanning and deployment of wing extension behaviors (recently reviewed in [19]), but it is not unexpected that it should have similar cuticle tanning roles prior to eclosion. Recent evidence has shown that a subset

of CCAP neurons (N_{CCAP}) which co-express bursicon undergo Ca^{++} changes in their cell bodies during pupal head eversion, suggesting that these neuropeptides are secreted at this point in ecdysis [20]. Notably, these same N_{CCAP} have axonal projections with type III boutons [5]. However, no study has quantified the secretion of neuropeptides from type III boutons in larval or pupal stages, or have tied the secretion of these hormones to the process of ecdysis.

In this study, we addressed the dual questions of secretory protein expression in type III boutons and the functional significance of their hormonal release at the NMJ in wild type, mutant, and transgenic *Drosophila* larvae. By limiting our scope to the peptidergic type III boutons we were able to focus solely on the requirements for neuropeptide release in *Drosophila*. The type III boutons are also unique as release sites at the NMJ, and we provide new insight into this conduit between the CNS and the periphery in the neuroendocrine regulation of a critical behavior.

2.3 Materials and Methods

Fly stocks

All stocks were reared on standard media at 25°C in 12h:12h LD cycle. Except where mentioned, all stocks are available from the Bloomington *Drosophila* Stock Center. The stock w^{1118} was used as the wild-type strain. $SNAP\text{-}25^{124}/Df(3L)1\text{-}16$ hemizygotes result in a SNAP-25 null phenotype, whereas SNAP-24 was

reduced in animals of the genotype *P[EPgy2]*^{EY10332}*/Df(3R)by10*. A stock of CCAP-GAL4 flies (gift of John Ewer) was used to drive expression in type III boutons of either UAS-ANF-EMD or UAS-Syx1A RNAi (Syx1A^{GD564}; Vienna *Drosophila* RNAi Center). Under our conditions, a strong phenotypic result was obtained by recombining UAS-Dicer2 (Vienna *Drosophila* RNAi Center) with CCAP-GAL4 to drive Syx1A RNAi. The *rickets* mutants *rk*^{1}*cn*^{1}*bw*^{1} and *rk*^{4} were used for phenotype comparisons of wings and legs. The enhancer trap *Syx1A*^{L247} was used to label nuclei of cell bodies in the CNS. Recombinants of *elav*-GAL4 and UAS-ANF-EMD were used as control animals for the CCAP>ANF-EMD release experiments.

Immunohistochemistry

Wandering 3rd instar larvae were dissected in cold Ca^{++} – free HL3 [21], filleted, and fixed for 2 hours – overnight in Bouin's fixative at room temperature. Fillets were thoroughly washed in PBSTx [PBS + Tween + 0.3% Triton X-100] and incubated in 10% - 20% normal donkey serum for 1 hour – overnight at 4°C. Tissues were then incubated in primary antibody overnight at 4°C, quickly rinsed and then washed 3 x 15 minutes in PBSTx. Subsequently, preparations were incubated for 2 hours – overnight in secondary antibody at 4°C, thoroughly rinsed and washed in PBSTx, and finally mounted on slides in Vectashield (Vector Laboratories) and stored in the dark at 4°C. Primary antibodies used include rabbit

anti-bursicon (1:5000; a generous gift from Ben White), rat anti-R29 (N-SYB) (1:200; a generous gift from Hugo Bellen), mouse anti-DCSP2 (CSP) (1:100; Developmental Studies Hybridoma Bank), guinea pig anti-1315 (SNAP-24/SNAP-25) (1:100), mouse anti-8C3 (Syx1A) (1:50; Developmental Studies Hybridoma Bank), goat anti-HRP (1:400; Jackson ImmunoResearch Laboratories), and mouse anti-β-galactosidase (1:500; Promega). For secondary antibodies, the relevant species was used with excitations of either 488 or 594 (1:1000; Invitrogen). For comparisons of wild type preparations to either reduced SNAP-24 or SNAP-25 null preparations, fillets were processed together in the same tubes of primary and secondary antibodies to ensure equal conditions.

Western blotting

Comparisons of relative levels of SNAP-24 and SNAP-25 were made between wild type (w^{1118}), reduced SNAP-24 ($P[EPgy2]^{EY10332}/Df$), and SNAP-25 null ($SNAP\text{-}25^{124}/Df$) flies. Western blots of tissue preparations from these genotypes (each consisting of five pharate adult heads) were probed with the 1315 antibody, following procedures previously described from our laboratory [22].

Histological preparation of legs

Legs were cleared by overnight incubation in 10% KOH at room temperature, dehydrated through an ethanol series and

mounted in Euparal (BioQuip Products, Inc.). Leg preparations were subsequently viewed on a Nikon Eclipse E600FN microscope at 10x and photographed with a SPOT2 camera (Diagnostic Instruments, Inc.) as 8-bit monochrome with the SPOT32 software (version 2.2), under equal exposure settings.

Imaging

Histological preparations were viewed on a Nikon Eclipse E600FN microscope at 40x, with the exception of leg preparations at 10x. Images were collected with a SPOT2 camera (Diagnostic Instruments, Inc.) as 8-bit monochrome with the SPOT32 software (version 2.2). Images of wild type, reduced SNAP-24, and SNAP-25 null preparations were collected with the same exposure settings. Images of adults in Figure 2.9 were taken on a Leica MZFLIII microscope and saved with Leica IM50 (version 1.20) software. All images were cropped and monochrome images were given their color identities with Adobe Photoshop CS (version 8.0). Figures were finalized with Adobe Illustrator CS (version 11.0).

Several images needed to be enhanced with ImageJ software (version 1.33u), due to either background signal or endogenous muscle staining. In Figure 2.7 we used an antibody that recognizes both SNAP-24 and SNAP-25 immunoreactivity at NMJ 12; our available SNAP-25 antibody was not compatible with the bursicon antibody. Aside from its role in vesicle release, SNAP-24 is also present in body wall muscle [22], which resulted in

low contrast between the boutons and muscle 12. We countered this problem by adjusting both brightness and contrast by equal levels for Figures 2.7B, 2.7E, and 2.7H. To improve the signal in our bursicon/Syx1A co-localization study (Figure 2.8), we utilized both the 'Subtract Background' and adjust brightness/contrast features.

Fluorescence intensity measurements

For the fluorescence measurements of ANF-EMD release from type III boutons in Figure 2.11, we chose six developmental stages that include the 2nd larval ecdysis. To obtain larvae approaching this ecdysis, it was necessary to collect CCAP>ANF-EMD embryos on grape juice plates with 3% agar, supplemented with yeast. The recognition of these stages are described in detail in the Results section. For each stage four animals were selected and all visible type III boutons were photographed with the same exposure setting. Since CCAP-GAL4-expressing boutons occur bilaterally in NMJ 12 of the T3-A4 segments, the maximum number of type III boutons that can be analyzed per animal is 5 pairs, although we were not always able to visualize this maximum number. With ImageJ software, we empirically determined a threshold at which we could select type III bouton area in images from the earliest time point (DMH larvae), and with this threshold value we measured mean pixel value in type III boutons at all stages. To calculate average fluorescence intensity, we multiplied

the area (selected by the threshold) and mean pixel value, and took the average of these values for each stage. Comparisons between stages to determine percent release were calculated as [fluorescence intensity$_0$]-[fluorescence intensity$_1$]/[fluorescence intensity$_0$]. Error bars represent the standard error of the mean (S.E.M.), but single-factor ANOVA tests were also performed to determine if the fluorescence intensity from adjacent stages differed significantly from each other. The single-factor ANOVA tests indicate strong significant difference between the following stages: DMH to DVP ($p=0.004$); FE to +2 hours ($p=0.0008$); +2 hours to +3 hours ($p=0.001$); and +3 hours to L3 ($p=3.4\times10^{-5}$). The one stage comparison without a significant difference is DVP to FE ($p=0.08$). As a control against large-scale vesicular release at the NMJ, the pan-neural *elav*-GAL4 driver was used to express ANF-EMD at all NMJs.

3.4 Results

Bursicon expression at the larval NMJ is limited to type III boutons

In *Drosophila*, bursicon is produced in a subset of N_{CCAP} in the CNS [19, 23, 24]. N_{CCAP} in the ventral ganglion, in T3 and A1-A4, send motor neuron axons to the type III boutons of NMJ 12 and 13 [5, 25]. Since CCAP and bursicon share overlapping expression patterns in the ventral ganglion it is likely that they are also co-expressed in type III boutons. We wished to use bursicon

immunoreactivity (BURS-IR) as a marker for type III boutons, but its expression pattern at the larval NMJ has not been shown with an independent marker for type III boutons. To verify if bursicon is expressed in type III boutons, we looked for its colocalization with an exogenous GFP marker driven by CCAP-GAL4. In this case, an emerald-GFP tagged atrial natriuretic factor (or UAS-ANF-EMD) construct was used as the marker for type III boutons. Labelling of CCAP>ANF-EMD larvae with an anti-rabbit bursicon antibody reliably demonstrated that BURS-IR (Figure 2.1A) and the targeted ANF-EMD expression pattern (Figure 2.1B) colocalize in type III boutons (Figure 2.1C). This pattern of colocalization suggests that the ectopic ANF-EMD protein is packaged with bursicon in the same vesicles. Furthermore, double-labelling with bursicon (Figure 2.2A) and HRP (Figure 2.2B) antibodies reveals that the bursicon hormone is only expressed in type III boutons at NMJ 12 (Figure 2.2C), whereas the other morphological bouton types fail to double-label with bursicon. Taken together, these data firmly establish that bursicon is expressed in type III boutons; all subsequent figures will use BURS-IR as a marker for type III boutons.

Expression of vesicle membrane proteins in type III boutons

We were curious whether the release of peptide hormones requires the same core exocytotic machinery as neurotransmitter release. Since it appears that the type III boutons are specialized for peptide hormone release, they could provide a model for

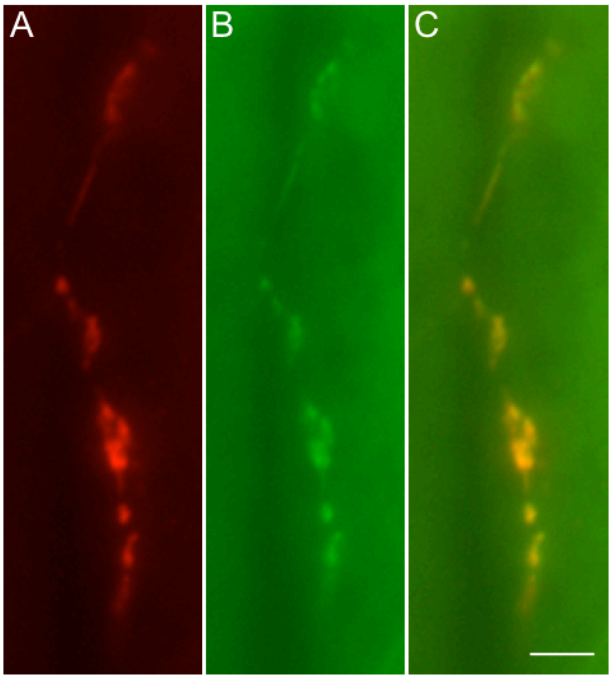


Figure 2.1 Endogenous bursicon colocalizes with targeted ANF-EMD in type III boutons. Transgenic larvae expressing the fluorescent neuropeptide marker ANF-EMD with a CCAP-GAL4 promoter were stained with an antibody recognizing the bursicon-α subunit, in order to verify the expression pattern of bursicon at the NMJ. (A) Bursicon immunoreactivity at a representative muscle 12. (B) Vesicles with ANF-EMD are distributed in type III boutons. (C) Bursicon protein colocalizes with the ectopic ANF-EMD marker. Scale bar = 10 μm.

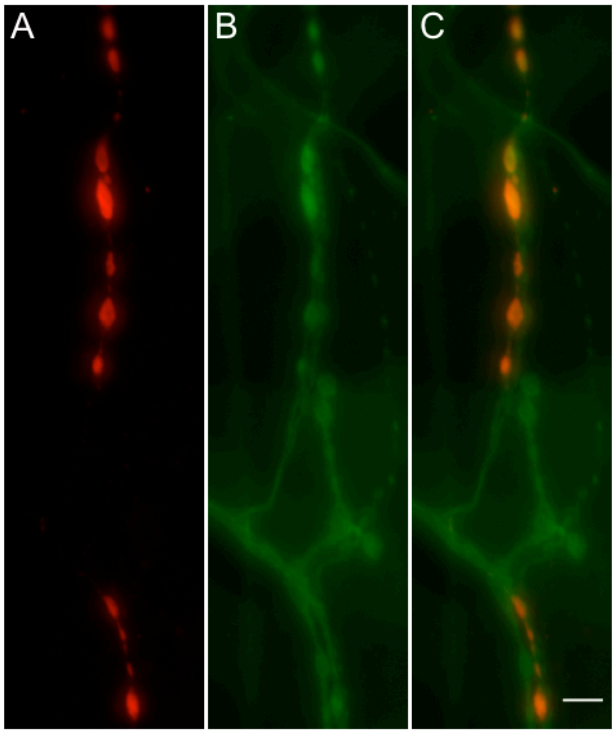


Figure 2.2 Buriscon immunoreactivity is limited to a subset of HRP-positive boutons. Type III boutons have a distinctive 'almond-shaped' morphology. While the pan-neural HRP marker could be used to morphologically distinguish type III boutons at the larval NMJ from type I and II, BURS-IR is a much more reliable marker. (A) Bursicon immunoreactivity as a marker for type III boutons. (B) HRP immunoreactivity labels all boutons at the represented muscle 12. (C) Bursicon distribution is found in some, but not all boutons at NMJ 12. Scale bar = 10 μm.

determining the specific synaptic proteins required for hormone secretion at the NMJ. We first examined the expression of vesicle membrane proteins believed to be required for exocytosis. Double-labelling of NMJ preparations with bursicon (Figure 2.3A) and N-SYB antibodies (Figure 2.3B) demonstrates that N-SYB is expressed in type III boutons (Figure 2.3C; arrows), as well as boutons which are devoid of BURS-IR (Figure 2.3C; arrowheads). Similarly, BURS-IR (Figure 2.4A) and CSP-IR (Figure 2.4B) colocalize in type III boutons (Figure 2.4C; arrows), with CSP-IR also occurring outside of the BURS-IR pattern (Figure 2.4C; arrowheads).

Localization of target membrane proteins in type III boutons

The target-membrane proteins SNAP-24 and SNAP-25 have been shown to subserve similar functions in vesicle release at the larval NMJ [22]. Using an antibody (referred to here as 1315, but previously referred to as Exon 4) that recognizes an identical region of the proteins SNAP-24 and SNAP-25 [22] in conjunction with the pan-neural HRP marker, we observed that at NMJ 12 (Figure 2.5A) the 1315-IR staining pattern (Figure 2.5B) appears to label all bouton types (Figure 2.5C). To determine which of these proteins may be required in the release of peptide hormones from type III boutons we examined 1315-IR staining patterns in animals with either reduced levels of SNAP-24 (*P[EPgy2]*^{EY10332}*/Df*) or in the complete absence of SNAP-25 (*SNAP-25*^{124}*/Df*), relative to wild

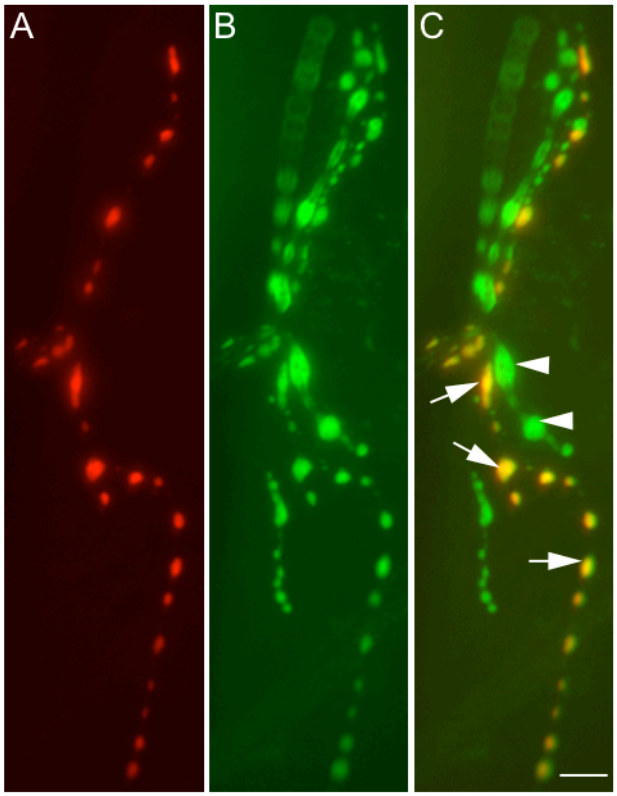


Figure 2.3 Neuronal synaptobrevin expression at NMJ 12 includes type III boutons. (A) Bursicon immunoreactivity in type III boutons. (B) Distribution of N-SYB immunoreactivity at NMJ 12. (C) N-SYB is expressed in multiple bouton types, including type III. Arrows indicate representative boutons that co-express bursicon and N-SYB, whereas arrowheads indicate boutons that do not express bursicon. Scale bar = 10 μm.

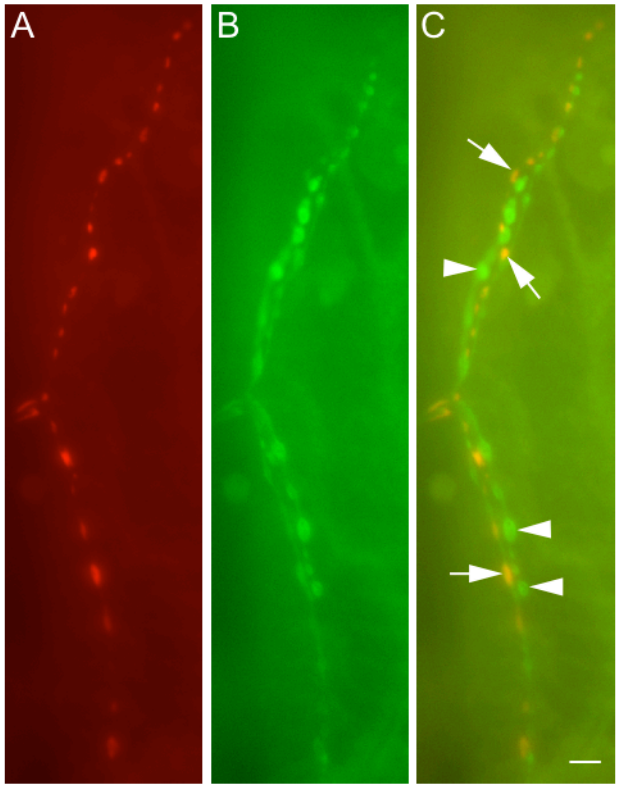


Figure 2.4 Cysteine string protein expression pattern at NMJ 12 includes type III boutons. (A) Bursicon immunoreactivity as a marker for type III boutons. (B) CSP immunoreactivity labels multiple boutons at NMJ 12. (C) Bursicon and CSP patterns colocalize in type III boutons. The expression of CSP immunoreactivity in type III boutons appears to be weaker than in other boutons at NMJ 12. Arrows indicate representative boutons that co-express bursicon and CSP, whereas arrowheads indicate boutons that do not express bursicon. Scale bar = 10 μm.

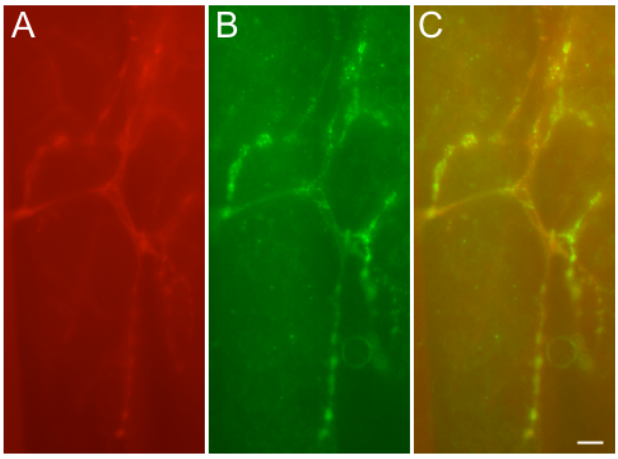


Figure 2.5 An antibody recognizing SNAP-24 and SNAP-25 labels all boutons at NMJ 12. (A) HRP immunoreactivity is distributed across all boutons at NMJ 12. (B) Staining pattern of 1315, an antibody recognizing an identical region of SNAP-24 and SNAP-25. (C) SNAP-24 and/or SNAP-25 expression in all boutons at NMJ 12 in wild-type larvae. SNAP-24 is also present in body wall muscles, which can diminish the contrast in staining between boutons and the muscle. Scale bar = 10 μm.

type controls. The proof of principle for this approach is illustrated in Figure 2.6: in a western blot of pharate adult head tissue blotted with the 1315 antibody, wild type animals (w^{1118}) express both proteins (lane A); *SNAP-25*^{124}*/Df* animals only express SNAP-24 (lane B); and *P[EPgy2]*^{EY10332}*/Df* animals express mostly SNAP-25, accompanied by a very reduced level of SNAP-24 (lane C). Comparisons of wild-type and reduced SNAP-24 NMJ preparations reveal that wild-type BURS-IR (Figure 2.7A) and 1315-IR (Figure 2.7B) colocalize in type III boutons (Figure 2.7C), whereas in animals with reduced SNAP-24, BURS-IR (Figure 2.7D) is still present yet 1315-IR (Figure 2.7E) is undetectable in type III boutons (Figure 2.7F). Our reciprocal experiment with SNAP-25 null mutants yields a similar result: animals deficient for SNAP-25 still express BURS-IR (Figure 2.7G), but 1315-IR (Figure 2.7H) is undetectable in type III boutons (Figure 2.7I). Since reductions of either SNAP-24 or SNAP-25 result in no detection by the 1315 antibody, this indicates that both proteins are present in type III boutons, but at a low level.

We also examined the expression of the target membrane protein Syx1A in type III boutons. Our available Syx1A antibody (8C3; Developmental Studies Hybridoma Bank) gave reliable results in the CNS, but staining of motor neuron terminals was qualitatively less reliable (data not shown). Nevertheless, we observed that BURS-IR (Figure 2.8A) and 8C3-IR (Figure 2.8B) shared colocalization in motor neuron axons projecting to type III boutons (Figure 2.8C). Additionally, in the ventral ganglion of

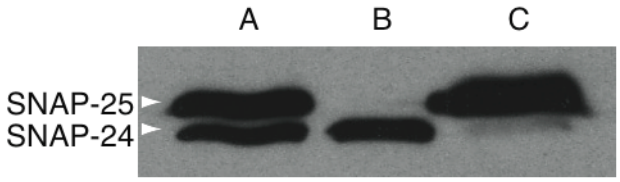


Figure 2.6 Expression of SNAP-24 and SNAP-25 proteins in wild-type or mutant animals. Western blot of pharate adult heads from wild-type (w^{1118}), SNAP-25 null ($SNAP\text{-}25^{124}/Df$) or reduced SNAP-24 ($P[EPgy2]^{EY10332}/Df$) flies probed with the 1315 antibody. Wild-type animals (lane A) show two bands, which correspond to the predicted weights of SNAP-24 and SNAP-25. SNAP-25 nulls (lane B) show one band corresponding to SNAP-24, and reduced SNAP-24 flies (lane C) have one band corresponding to SNAP-25 and a faint residual band corresponding to SNAP-24.

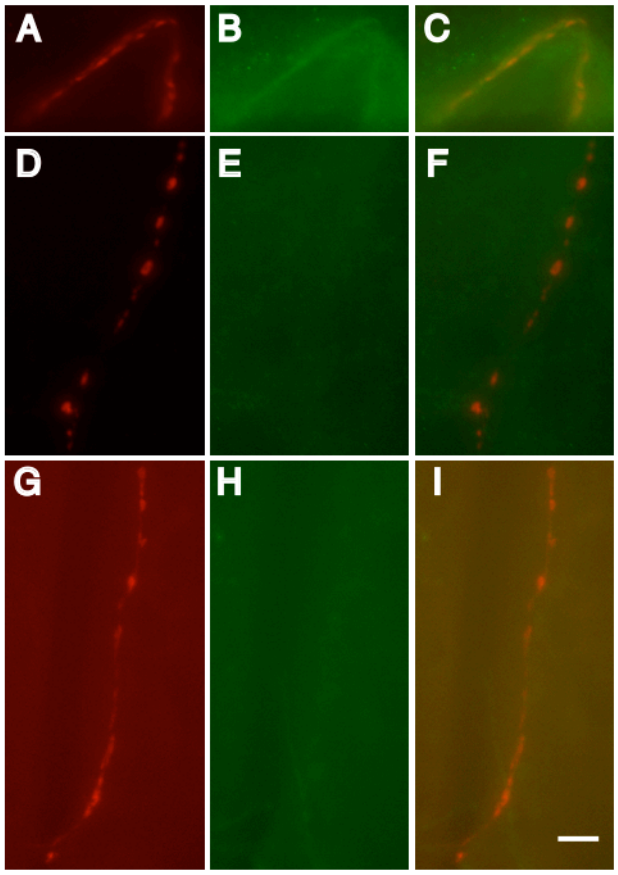


Figure 2.7 Expression of SNAP-24 and SNAP-25 in type III boutons. We double-labeled wild-type (A-C), reduced SNAP-24 (D-F), and SNAP-25 null (G-I) larval NMJs with bursicon and 1315 antibodies. (A, D, G) Bursicon immunoreactivity in type III boutons. (B, E, H) NMJ 12 stained with 1315 antibody. (C) In wild-type (w^{1118}) animals, 1315 immunoreactivity distributes with bursicon in type III boutons. (F) Reduced SNAP-24 mutants ($P[EPgy2]^{EY10332}/Df$) show bursicon immunoreactivity in type III boutons with no accompanying 1315 immunoreactivity. (I) SNAP-25 nulls ($SNAP\text{-}25^{124}/Df$) express bursicon in type III boutons with no accompanying 1315 immunoreactivity. Scale bar = 10 μm.

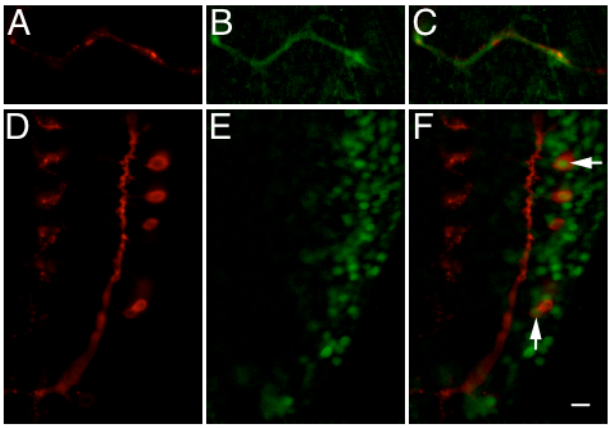
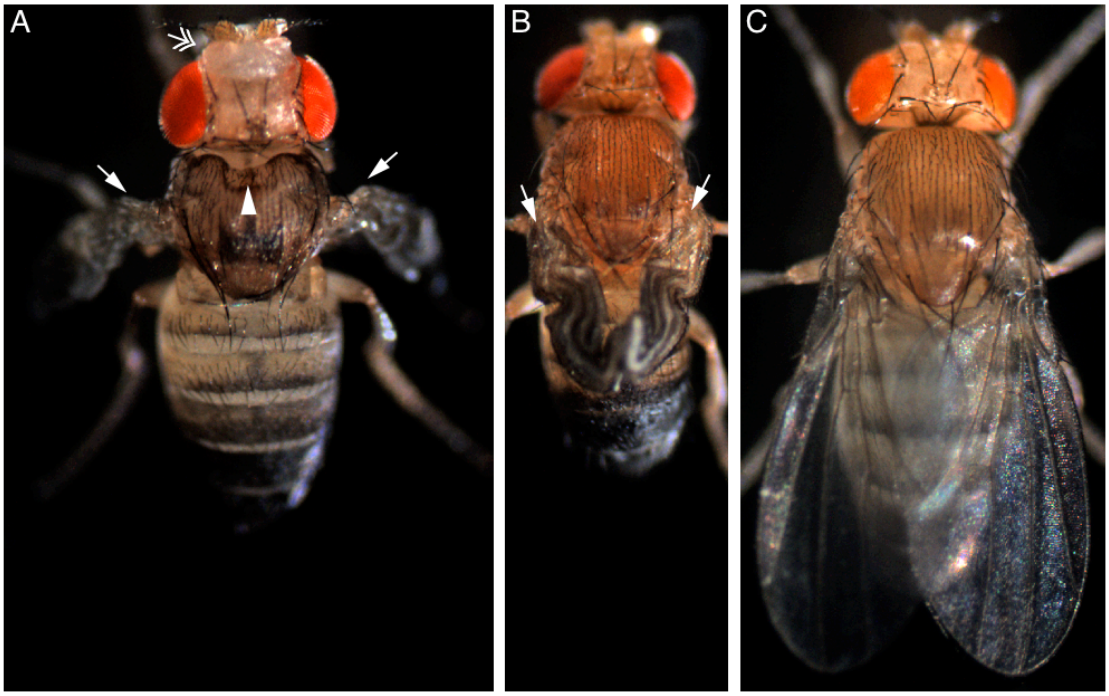


Figure 2.8 Co-expression of Syxntaxin 1A and bursicon in axons and the ventral ganglion. (A-C) Using bursicon and Syx1A (8C3) antibodies, we observed the co-expression of bursicon and Syx1A in motor neuron axons. BURS-IR (A) and 8C3-IR (B) are co-expressed in a motor neuron axon projecting towards NMJ 12 (C). (D-F) Additionally we observed the co-expression of bursicon with a nuclear-localized *Syx1A* enhancer trap, in N_{CCAP} in the ventral ganglion which project axons with type III boutons. In the CNS of a $Syx1A^{L247}$ larva, BURS-IR in the ventral ganlion (D) labels the cell bodies of N_{CCAP}, whereas β-galactosidase staining labels the nuclei of neurons expressing Syx1A (E), including the BURS-positive NCCAP (F). Arrows indicate representative N_{CCAP} expressing both bursicon and Syx1A. Scale bar = 10 μm.

larvae with a nuclear-localized *Syx1A* enhancer trap (*Syx1A*^{L247}), cell bodies labelled with BURS-IR (Figure 2.8D) also express *lacZ* (Figure 2.8E), in the N_{CCAP} which send axons to NMJ 12 (Figure 2.8F). In spite of the performance of the 8C3 antibody at the NMJ, we believe these observations together establish that Syx1A is expressed in type III boutons.

Could reduction of expression of Syx1A hinder the release of vesicles from type III boutons? We used a UAS-Syx1A RNAi construct to perturb expression of Syx1A in type III boutons. To test the efficacy of the UAS-Syx1A RNAi we crossed it initially to an Act-5c GAL4 line which expresses GAL4 ubiquitously at high levels. We observed no hatched embryos from this cross (data not shown), suggesting that the Syx1A RNAi construct is effective at blocking synaptic transmission (cf. [26, 27]). Since we had no way to limit transgenic expression to the type III boutons, we decided to use the CCAP-GAL4 driver which expresses in these same boutons *in addition to* other N_{CCAP}. The genotype CCAP>Syx1A RNAi did not show any observable mutant phenotypes, but the addition of UAS-Dicer2 to increase the RNAi effect [28] resulted in all progeny displaying a range of phenotypes that paralleled mutations in the bursicon receptor *rickets* (cf. [29]). These CCAP>Dicer2 + Syx1A RNAi progeny (Figure 2.9A) had unextended wings (arrows), cuticular dimpling on the anterior dorsal thorax (arrowhead), and a persistently un-tanned ptilinum (feathered arrowhead). Unextended wings are a hallmark of the bursicon receptor mutants *rk*^{4} (Figure 2.9B, arrows) and *rk*^{1} (data

Figure 2.9 Disruption of hormone secretion from N_{CCAP} with Syx1A RNAi results in juvenile adult phenotypes. We drove UAS-Syx1A RNAi and UAS-Dicer2 with the CCAP-GAL4 promoter to examine the role of Syx1A in peptide hormone secretion from N_{CCAP} (including the type III boutons). Disruptions in the bursicon signaling pathway are known to result in unextended wings, and tanning defects. The Syx1A RNAi knock-down progeny (A) exhibited several freshly emerged adult phenotypes such as an un-tanned ptilinum (feathered arrow) and a dimpled thorax (arrowhead). A distinctive characteristic found in common between our Syx1A RNAi knock-down flies and the *rk*^{4} allele (B) was the presence of unextended wings (arrows). The angle at which *rickets* mutants hold their wings can be somewhat variable, but it appears to be distinct from the pronounced downward angle seen in (A). We observed no differences between the UAS-Syx1A RNAi stock (C), the CCAP+Dicer2 recombined stock, and wild-type controls (data not shown).


A
B
C

not shown), as are cuticular defects related to incomplete tanning. None of these juvenile phenotypes are observed in a 'wild type' UAS-Syx1A RNAi control (Figure 2.9C). Additionally, CCAP>Dicer2 + Syx1A RNAi flies showed greatly bowed metathoracic tibiae (Figure 2.10A, arrow), as compared to the strong rk^{1} mutant allele (Figure 2.10B, arrow). In contrast, the less severe rk^{4} allele (Figure 2.10C) and the UAS-Syx1A RNAi control (Figure 2.10D) did not exhibit these pronounced tibial deformities. The combined Syx1A RNAi phenotypes documented in Figures 2.9 and 2.10 strongly implicate a bursicon release deficiency in CCAP>Dicer2 + Syx1A RNAi flies. We believe that these results confirm an essential role for Syx1A in the release of bursicon.

Secretion of ANF-EMD from type III boutons before and after ecydsis

What is the functional significance of the type III boutons as a peripheral release site? Hormones such as CCAP, MIP and bursicon that are expressed in these peptidergic terminals have roles in ecdysis and post-ecdysis events. One approach to elucidate the role of the type III boutons is to determine when they release their hormone stores, and attempt to correlate this with a relevant native event such as ecdysis. Rather than examining BURS-IR at different developmental stages *in situ*, we decided to image live transgenic larvae that expressed the GFP-tagged neuropeptide reporter UAS-ANF-EMD in the CCAP-GAL4 pattern.

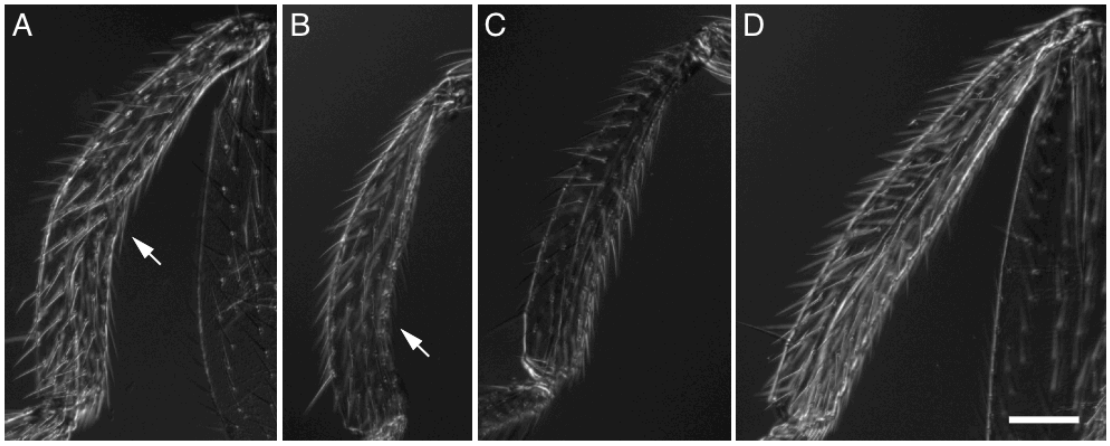
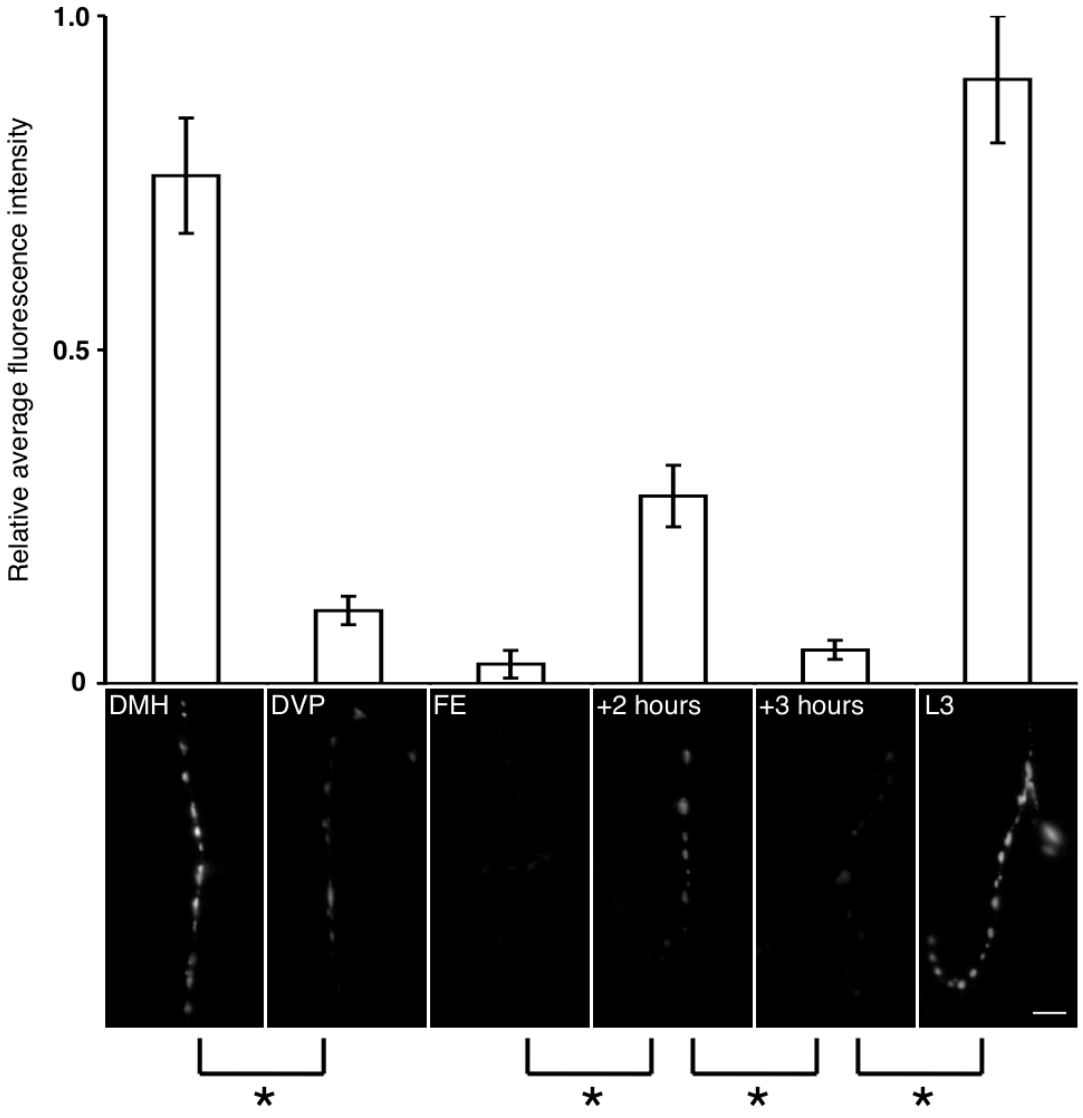


Figure 2.10 Targeted expression of Syx1A RNAi causes a bowed leg phenotype. The bursicon receptor mutants *rk*^{1} and *rk*^{4} can display a range of variable leg deformities, including bowed femurs and tibiae, and flattened and/or kinked tarsi. In our Syx1A RNAi knock-down flies (CCAP-GAL4 driving UAS-Syx1A RNAi + UAS-Dicer2) we observed a pronounced mid-tibial bow in the metathoracic leg of all progeny (A). This effect was similar to, but more severe than, tibial bowing observed in *rk*^{1} animals (B). In contrast, the *rk*^{4} allele (C) was indistinguishable from the UAS-Syx1A RNAi stock (D), the CCAP+Dicer2 recombined stock, and wild-type controls (unpublished observations). Arrows indicate tibial bowing in (A) and (B). Scale bar = 0.1 mm.

The ANF-EMD construct [30] has been used successfully on several occasions to monitor vesicle release from secretory cells during endogenous behaviors (as documented in the discussion section).

While not directly addressing the question of endogenous hormone release, this technique allowed us to accurately quantify when the type III boutons release ANF-EMD, by directly measuring fluorescence intensity fluctuations. We initially examined fluctuations in fluorescence intensity in the NMJs of CCAP>ANF-EMD animals approaching the second larval ecdysis, and those which had begun secreting ETH, which is believed to be the initial peptide hormone trigger of ecdysis behaviors [31]. These two stages can be distinguished on the basis of mouthpart morphology known as 'double mouth hooks' (DMH) and 'double vertical plates' (DVP), respectively [31]. Imaging of the DMH stage indicated high levels of ANF-EMD in type III boutons (Figure 2.11). Less than two hours later, at the DVP stage, there is an 85% decrease in fluorescence intensity, indicating large-scale secretion. This is a startling result, since no ecdysis-related peptide hormone is currently known to precede the release of ETH at the DVP stage. This wave of ANF-EMD release continues through the end of ecdysis behaviors, until when the 3rd instar larva finally breaks through the old cuticle with a thrusting forward escape (FE) manoeuvre [31]. The level of ANF-EMD release at FE represents its lowest expression level, a 96% drop in fluorescence intensity from the initial DMH levels. Following this stage, type III boutons

Figure 2.11 Two phases of vesicle release overlap with larval ecdysis. We observed fluorescence changes in the type III boutons of CCAP>ANF-EMD larvae. Six stages were chosen that broadly extend over the duration of the 2nd larval ecdysis: 'DMH', which precedes the onset of ecdysis; 'DVP', which coincides with ETH release; 'FE', which signifies the completion of ecdysis; '+2 hours' after FE; '+3 hours' after FE; and 'L3', wandering 3rd instar larvae. At each stage, the fluorescence intensity of all visible type III boutons from 4 animals was measured and converted to fluorescence intensity averages (see Methods), in arbitary units (top panel). Representative type III boutons from each stage are shown below the corresponding fluorescence intensity measurements with accompanying asterisks representing single-factor ANOVA results for consecutive stages (bottom panel). The results significantly show two waves of ANF-EMD release, before DVP and after FE, as shown by an asterisk. For *, $p<0.005$. Error bars indicate +/- s.e.m. Scale bar = 10 μm.


1.0
0.5
0
Relative average fluorescence intensity
DMH
DVP
FE
+2 hours
+3 hours
L3
*
*
*
*

appear to refill with ANF-EMD. Measurements taken two hours after FE have rebounded to a level representing 37% of initial DMH levels, suggesting that refilling has begun (see +2 hours, Figure 2.11). However, within one hour we detected a second decline in fluorescence intensity in type III boutons: at three hours post FE (see +3 hours, Figure 2.11) approximately 82% vesicle release has occurred, relative to the preceding stage. Thus there appears to be two distinct waves of ANF-EMD release from type III boutons, the first preceding ETH release at DVP and the second following the completion of ecdysis behaviors at FE. By the wandering 3rd instar stage, the fluorescence intenstiy has returned to levels seen before ecdysis (see L3, Figure 2.11). As a control we also examined larvae expressing ANF-EMD with a pan-neural driver, *elav*. Examination of normally non-peptidergic type I boutons on NMJ 6/7 between DMH and DVP revealed no noticeable change in fluorescence intensity (data not shown). This indicates that the changes in the type III boutons are not the result of large-scale synaptic activity at the NMJ.

The nature and identity of hormone release from type III boutons occurring before DVP will require further probing since we limited our study to ectopic ANF-EMD expression and release. The second wave of ANF-EMD secretion (post-FE) could correspond to the release dynamics previously observed to following pupal ecdysis [20].

3.5 Discussion

Synaptic proteins that make up the exocytotic machinery are essential for proper evoked release of neurotransmitters. Remarkably, there is little *in situ* evidence of a similar role for these proteins in neuropeptide secretion, despite the important regulatory and modulatory roles these hormones have on a diverse array of physiology and behaviors. We addressed this issue by looking at the distribution of the core SNARE proteins and CSP in the uniquely peptidergic type III boutons of the *Drosophila* larval NMJ. Not surprisingly, the synaptic proteins that we observed in type III boutons have previously been shown to be required for neurotransmitter release. Thus there is likely a fundamental requirement for SNARE protein-protein interactions between the vesicle and the target membrane to liberate vesicle contents. However, expression patterns do not reveal function, and only in a genetically tractable organism, such as *Drosophila*, can these expression patterns be perturbed to identify how release is compromised.

In our preliminary experiments we were unable to disrupt the expression of either N-SYB or CSP using targeted RNAi (data not shown), but our functional experiments with SNAP-24, SNAP-25 and Syx1A reveal some interesting roles for these proteins. Regarding SNAP-24 and SNAP-25, previous results from our group had shown that these two proteins are co-expressed at the NMJ [22]. In a SNAP-25 null background, SNAP-24 can provide a rescue function until the pharate adult stage. These SNAP-25

mutants fail to eclose, although it is currently unclear if this is related to an inability to secrete hormones that initiate ecdysis or to broader problems brought on by a complete lack of SNAP-25. What it does suggest though is that SNAP-25 is essential for late pupal development and that SNAP-24 substitution at this stage is ineffective. In contrast, reduced SNAP-24 mutants in the current study are viable as adults, none of which exhibit any phenotypes associated with *rickets* mutants (data not shown). Perhaps the dual expression of SNAP-24 and SNAP-25 in the type III boutons is to ensure that a vital function subserved by their neuroendocrine release is successfully executed.

We observed bursicon and Syx1A colocalization in N_{CCAP} in the ventral ganglion and motor neuron axons, a pattern which we could not determine in the type III boutons. In support of a role for Syx1A in bursicon release, we found that transgenic expression of UAS-Syx1A RNAi with CCAP-GAL4 (and UAS-Dicer2) was sufficient to phenocopy many hallmarks of both bursicon mutants [23] and *rickets* mutants [29]. These include unextended wings, untanned cuticle with dimpling, and bowed legs. However, *rickets* mutants are smaller and the angle at which they hold their wings differs from our RNAi knock-down flies. Are these transgenically induced phenotypes a direct result of Syx1A reduction in N_{CCAP}? There is a possibility that the inclusion of UAS-Dicer2 to obtain these phenotypes could have increased the chance of off-targeting effects [28], vis-à-vis up-regulation of a processing step in the production of endogenous micro-RNA duplexes [32] in the N_{CCAP}.

Indeed, in our recombinant CCAP-GAL4 + UAS-Dicer2 stock, 6% of the population show an unextended wing phenotype in the absence of any transgenic RNAi expression, but bowed leg segments are never observed. Considering the restrictive CCAP-GAL4 pattern and the 100% penetrance of *rickets* phenotypes in both wings *and* legs, we believe that the expression of Syx1A in N_{CCAP} has been compromised in our RNAi knock-down flies, leading to suppressed bursicon release.

We have confirmed bursicon expression in larval type III boutons by its colocalization (but see also [33]) with transgenic ANF-EMD driven by CCAP-GAL4. We could have used immunohistochemistry to one of the hormones present in type III boutons to study its timing of release, but this method is difficult to perform quantitatively. Instead our approach was to use the UAS-ANF-EMD construct, which codes for the rat ANF prepropeptide tagged with the emerald variant of GFP [30]. Importantly, this ectopic neuropeptide also has no biological activity in *Drosophila*. The ANF-EMD marker has been used on numerous occasions to monitor vesicular release in *Drosophila* [34-39], and has become an effective method to study hormone release during an endogenously performed behavior [40-43]. Of these behavioral studies, one of the most convincing uses of UAS-ANF-EMD focused on its release from N_{CCAP} in the ventral ganglion [40]. Preparations were compared at times before and after the 2nd larval ecdysis, and the decrease in ANF-EMD fluorescence corresponded with the previously observed decrease in CCAP immunoreactivity

between these same stages [18]. Thus measuring fluorescence dynamics in cells expressing UAS-ANF-EMD is a reliable method for studying the relationship between a behavior and the underlying hormonal release.

The expression of both CCAP and bursicon in the type III boutons should indicate the importance of the type III boutons to ecdysis-related release. Surprisingly, this function has not been previously addressed, even though type III boutons represent a neuroendocrine link between the CNS and the periphery. We measured ANF-EMD release at multiple stages before, during and after the 2nd larval ecdysis, representing *in toto* hormonal release from the type III boutons. Our results indicating ANF-EMD release in the hours following ecdysis are consistent with putative release timing [20] and a role for bursicon in tanning the new cuticle [19]. Strikingly, we also observed a significant amount of ANF-EMD release earlier, extending from between the DMH and DVP stages to the end of ecdysis. At the 2nd larval ecdysis, the DMH stage can precede DVP (when ETH is first released) by up to 2 hours with the end of ecdysis occurring approximately 35 minutes after DVP (see [31]).

We believe that the ANF-EMD fluorescence dynamics initiated between DMH and DVP stages represent an as-yet unidentified hormonal signal at the NMJ, preceding ETH release. This is in direct contrast to the current model of the neuroendocrine regulation of ecdysis, which places ETH release at the top of the sequence [44]. It is not clear if this pre-DVP hormone release

could be part of the actual neuroendocrine cascade that regulates ecdysis behaviors, or if it has another function. In support of a role for pre-DVP hormonal secretion from type III boutons, 2nd instar larvae with cell-ablated N_{CCAP} (accomplished by CCAP-GAL4 driving UAS-*reaper*) are able to successfully perform ecydsis behaviors, yet the duration of muscular contractions which are employed to shed the old cuticle are significantly longer [17]. In the context of these results, one role for our observed pre-DVP hormonal release from type III boutons could be to "prime" the NMJ for the ensuing events.

Importantly, no function has previously been ascribed to the type III boutons. Our results clearly indicate that they express the same core synaptic proteins as are required for neurotransmitter release. We found that hormonal release corresponds with a developmental stage when larvae perform ecdysis, suggesting a function for release from type III boutons. By using transgenic larvae expressing a GFP-tagged neuropeptide we were able to detect a previously unknown wave of neuropeptide release that precedes the initiation of ecdysis behaviors. These results underscore the need to reconsider the temporal and spatial roles of neuropeptides which regulate this essential insect behavior.

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CHAPTER 3

Tissue-specific requirements for rickets receptor expression during *Drosophila* development

3.1 Abstract

In *Drosophila*, the neuropeptide bursicon regulates wing expansion behaviors and cuticular tanning following adult eclosion. These dual processes are strikingly impaired in the viable rk^1 and rk^4 mutants, alleles of the bursicon receptor, *rickets*. To determine where *rickets* expression is required to mediate these post-eclosion events, in addition to probing for rickets activity in early development, we have transgenically silenced *rk* expression by RNA interference (RNAi) targeted to different tissues. Although strong ubiquitous expression of *rk* RNAi is developmentally lethal, this is surprising since rk^1 and rk^4 were thought to be nulls, and both alleles are homozygous viable. Here we show that these mutants behave genetically like severe hypomorphs, not null mutants. By narrowing down *rickets* RNAi expression to more selective tissue patterns, we provide evidence that *rickets* expression is required in the central nervous system for wing expansion behaviors, and in the imaginal discs for cuticular tanning. Quite unexpectedly, we found that *rickets* expression is obligatory in imaginal discs and somatic epidermis, prior to eclosion. Strong imaginal disc-specific expression of *rickets* RNAi prevents the pharate adult legs from obtaining sufficient rigidity needed for eclosion movements, whereas *rickets* RNAi targeted to

epidermal tissue terminally results in small, misshapen puparia that fail to tan. Finally, we show that these developmental prerequisites for *rickets* coincide with contemporaneous secretion of a GFP-tagged neuropeptide reporter from two subsets of bursicon-releasing neurons. Our results indicate essential and unexpected roles for the bursicon signaling pathway, at developmental stages previously unexamined.

3.2 Introduction

Upon completion of their final ecdysis (eclosion), adult *Drosophila* emerge with a soft cuticle and unexpanded wings. While these features are advantageous for confinement within an otherwise too small puparium, they are not adaptive to life outside of the puparium. Instead, shortly following eclosion the wings expand to their full size and the new cuticle undergoes sclerotization and melanization (tans) (see [1] for a recent review).

Several key observations provide evidence that these post-eclosion events are regulated by the neuropeptide bursicon through its receptor, rickets. First, the *rickets* mutants rk^{1} and rk^{4} are incapable of wing expansion behaviors or tanning their cuticle, even when challenged with hemolymph extracts containing bursicon from newly eclosed wild-type flies [2]. Similarly, *bursicon* subunit mutants are unable to successfully execute this phase of post-eclosion development [3]. The demonstration that bursicon hormone (in its active heterodimeric form) activates the rickets receptor, by increasing downstream cAMP activity [4, 5], completes

the link between hormone signaling and post-eclosion development.

A role for the rickets receptor apart from post-eclosion development has been more difficult to demonstrate, primarily because rk^1 and rk^4 exhibit few if any developmental defects prior to eclosion (cf. [2]). However, this does not preclude the bursicon signaling pathway from performing similar cuticle tanning roles following larval ecdyses and pupal ecdysis. In support of this possibility, transcripts of *rk* and the bursicon subunits *burs* and *pburs* are found at all stages of post-embryonic development [4, 5] (summarized in [6]). Furthermore, burs subunit immunoreactivity is present in several larval tissues, including CNS [4, 7] and select motoneuron terminals at the neuromuscular junction (NMJ) (Chapter 2 of this dissertation, in addition to [7]). Finally, we have recently determined that these terminals (or 'type III boutons') at the NMJ secrete hormones in two phases, both preceding and following the completion of the second larval ecdysis (Chapter 2 of this dissertation). Although we examined the release of an ectopic GFP-tagged neuropeptide (ANF-EMD) from these terminals, the type III boutons endogenously express neuropeptides implicated in ecdysis and post-ecdysis, including crustacean cardioactive peptide (CCAP) [8, 9] in addition to bursicon.

The expression and likely release of bursicon during development are strong indicators of other functions apart from post-eclosion events. However, these may not be easily deducible with the available *burs* and *rk* mutants, none of which result in a

complete absence of gene product (cf. [2, 3]). On the other hand, several transgenic studies hint at the possibility that disrupting bursicon signaling during development can have lethal consequences. Ectopic expression of the cell death gene *reaper* in CCAP neurons [10] results in two classes of progeny: those flies which eclose exhibit cuticular deformities and unexpanded wings remarkably similar to those of *burs* and *rk* mutants. Flies in the other class of progeny are incapable of performing head eversion, a crucial event at pupal ecdysis, and subsequently die as pupae. The defects at head eversion are even more frequent when CCAP neuron membrane excitability is suppressed by transgenic expression of a human K^+ channel inward rectifier (UAS-*Kir2.1*) [11]. Nevertheless, this pupal lethal phenotype should be interpreted with the caveat that CCAP neurons express multiple neuropeptide identities, including CCAP, bursicon and myoinhibitory peptide [12], which may obscure the determination of the head eversion defect. Thus, neither genetic mutants nor cell ablation studies are reliable means to elucidate the function of bursicon signaling during development.

Alternatively, the function of bursicon/rickets during development could be examined with RNA interference (RNAi). This method takes advantage of the endogenous cellular mechanisms to recognize double-stranded RNA (dsRNA) and abolish any identical native transcripts: by introducing transcript-specific dsRNA, the expression of a gene of interest can be silenced (cf. [13]). Surprisingly, this technique has not yet been

used to experimentally manipulate *burs* or *rk* expression in *Drosophila*. In this study we address the role of bursicon hormone at its target tissues during development, by restricting the expression of *rickets* with RNAi.

3.3 Materials and Methods

All fly stocks were raised on standard media and maintained at room temperature (25ºC). Where required, embryos were collected on grape juice plates (3% agar) augmented with yeast to facilitate collection of early larval instars. Stocks are available from the Bloomington *Drosophila* Stock Center (accompanied by stock number), except where noted otherwise. In the text, 'wild-type' refers to w^{1118}.

Transgenic fly stocks and phenotypic characterizations

Targeted RNAi phenotypes were obtained by crossing the GAL4 drivers Act5C(II) (#4414), Act5C(III) (#3954), T76 (#6995), T80 (#1878), 69B (#1774), C855a (#6990), and MJ33a (#6992) to UAS-*rk* RNAi ($rk^{GD14383}$; Vienna *Drosophila* RNAi Center). Progeny from these crosses were viewed with a Leica MZFLIII microscope and photographed with Leica IM50 (version 1.20) software. The GAL4 expression patterns of these drivers were then confirmed when crossed to UAS-*mCD8::GFP* (#5137). GFP-fluorescing 3rd instar larvae were dissected in Ca^{++}-free HL3 saline [14] and viewed as live preparations on a Nikon Eclipse E600FN microscope at 40x. Preparations were photographed with a

SPOT2 camera (Diagnostic Instruments, Inc.) as 8-bit monochrome with the SPOT32 software (version 2.2), under equal exposure settings. Mutant phenotypes were not observed when UAS-*rk* RNAi was crossed to the following GAL4 drivers: *elav* (#458), CCAP (gift from John Ewer), c929 [15], 386 [16], n-syb (gift from Julie Simpson), 36y (gift from Paul Taghert), r^4 (gift from Jae Park), and FB (gift from Thomas Neufeld).

Analysis of homozygous and hemizygous rickets phenotypes

The classic *rickets* alleles rk^1 cn^1 bw^1 (#3589) (referred to as rk^1 in the text) and rk^4 (#3590) were used in this study. These mutants were individually crossed to the deficiency *Df(2L)BSC252/CyO* (#23152), which deletes the *rickets* gene in addition to adjacent genomic regions [17]. Adult metathoracic legs were surgically removed from homozygous rk^1 and rk^4, as well as hemizygous rk^1/Df and rk^4/Df. Legs were histologically prepared and viewed as described in Chapter 2 of this dissertation. For convenience, we narrowed our phenotypic observations to the metathoracic tarsi, although it was not uncommon for additional leg segments to also show deformities. We classified tarsal phenotypes into three categories of increasing severity and scored them as either resembling wild-type; exhibiting a kink in the 1st tarsal segment; or exhibiting a bulbous 2nd tarsal segment (T2) with rotated distal tarsal segments, in addition to a T1 kink.

For homozygous and hemizygous rk^1 lethality comparisons, we created a stock of *Df(2L)BSC252* balanced with *CyO, ActGFP*

(#4533) to facilitate the identification of pupae hemizygous for the deficiency.

Assay of rickets transcript levels by reverse-transcription PCR

RT-PCR was used to assay *rickets* levels in wild-type (w^{1118}) CNS, fat body, imaginal discs, and body wall (with attached epidermis). Ten 3rd instar larvae were dissected in Ca^{++}-free HL3 saline and tissues were harvested and individually homogenized in TRIZOL Reagent (Invitrogen). After isolation of total RNA (following the supplied Invitrogen protocol), samples were reverse-transcribed according to the manufacturer's instructions with SuperScript III enzyme (Invitrogen). Primers specific to the *rickets* gene were used to amplify cDNA templates. The forward primer sequence was 5'-CATACACAAGGAAGCCTTTTCC-3' and the reverse primer sequence was 5'-CGGCAAACTTCCTGTAGTCC-3'. The following program was used to amplify the product. The initialization step was 1 cycle of 94°C for 3 minutes; followed by 40 cycles of 94°C for 15 seconds, 48°C for 30 seconds, 72°C for 1 minutes; with a final elongation step of 1 cycle of 72°C for 5 minutes. PCR products were analyzed by agarose gel electrophoresis (1.2% agarose in 1X TAE) and visualized with ethidium bromide staining under UV fluorescence.

Live imaging of CCAP>ANF-EMD pupae

A GFP-tagged 'atrial natriuretic factor' reporter, UAS-ANF-EMD (#7001) [18] was expressed with CCAP-GAL4 to monitor *in*

vivo neuropeptide release from bursicon-releasing CCAP neurons in CCAP>ANF-EMD progeny. Pupae were harvested from the culture vial walls and staged according to standard morphological characters associated with their development [19]. We assayed stages from initial puparium formation through the completion of pupal ecdysis. Staged pupae were dissected in Ca^{++}-free HL3 saline with a dorsal – longitudinal incision and splayed open with pins on magnetic plates. ANF-EMD fluorescence was observed and recorded in all preparations as previously described in Chapter 2 of this dissertation. In Figure 3.8, fluorescence intensity was calculated as described in Chapter 2 of this dissertation. For each time point, all observable type III boutons were measured from two pupae. Tracking release of the ANF-EMD reporter from discrete animals for each time point has been performed with success in the past (cf. [20]). Due to issues with background level, we were unable to take reliable measurements from the CCAP neuron arborizations in the CNS, in Figure 3.7.

3.4 Results

Ubiquitous expression of UAS-rk RNAi diminishes rickets transcript levels

We previously examined neuropeptide release from type III boutons at the larval NMJ (Chapter 2, this dissertation), which express both CCAP [8, 9] and bursicon (Chapter 2 of this dissertation; [7]). Transgenic ablations of these neurons have interesting consequences, one of which is that adult progeny

phenocopy *burs* and *rk* mutants (cf. [10, 11]). This provoked us to consider where *rk* expression could be functionally relevant during earlier stages of *Drosophila* development. We chose to answer this question by expressing *rk* RNAi widely and screening for resultant phenotypes.

We initially drove expression of UAS-*rk* RNAi with either of two separate Act5C-GAL4 insertions (on chromosome II or III). When driven with the 2nd chromosome Act5C driver, Act5C(II)>*rk* RNAi progeny primarily die at the pupal stage (data not shown). The few progeny which eclose resemble *rk* mutants in that they are unable to tan their cuticle or expand their wings (Figure 3.1A). Contrary to classic *rk* phenotypes, these flies have great difficulty standing. This is a likely consequence of extreme cuticular defects, and all adults die within 24 hours of eclosion. A greater developmental lethality results when UAS-*rk* RNAi is driven with Act5C(III)-GAL4. These progeny are unable to develop beyond the larval stage, and the few larvae which develop to the 3rd instar often die with a 'double vertical plates' phenotype (Figure 3.1B), indicative of failed larval ecdysis (cf. [21]). These results are consistent with a previous report that the same UAS-*rk* RNAi construct is 100% lethal when ubiquitously expressed with Act5C-GAL4 (see Supplementary Table 4, [13]). However, these RNAi phenotypes were not described, nor was the underlying basis explored at the time.

The rickets mutations rk^{1} and rk^{4} are more severe as hemizygotes

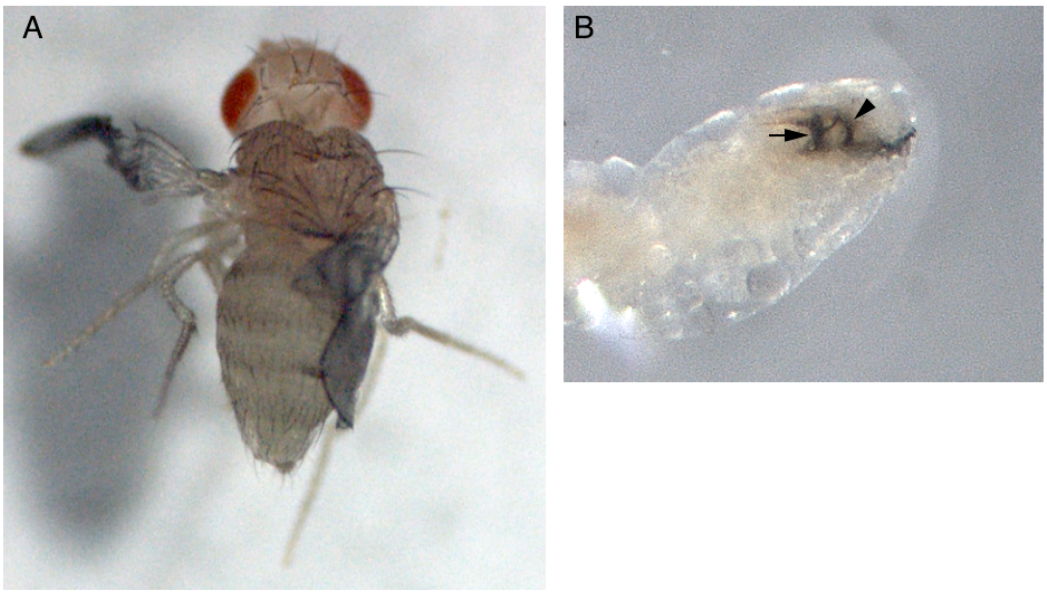


Figure 3.1 Lethality resulting from ubiquitous expression of UAS-*rk* RNAi. Two different Act5C-GAL4 lines result in lethality in combination with UAS-*rk* RNAi. (A) Although most progeny die as pupae (data not shown), Act5C(II)-*rk* RNAi adults occasionally eclose. Their wings never expand and their cuticle never tans. These adults are so weak that they die within 24 hours. (B) Act5C(III)-*rk* RNAi progeny all die before the end of the 3rd larval stage. These larvae often exhibit the double vertical plates phenotype, indicative of failed larval ecdysis. New 3rd instar mouthparts are labeled with the arrow; the 2nd instar mouthparts which failed to shed are labeled with the arrowhead.

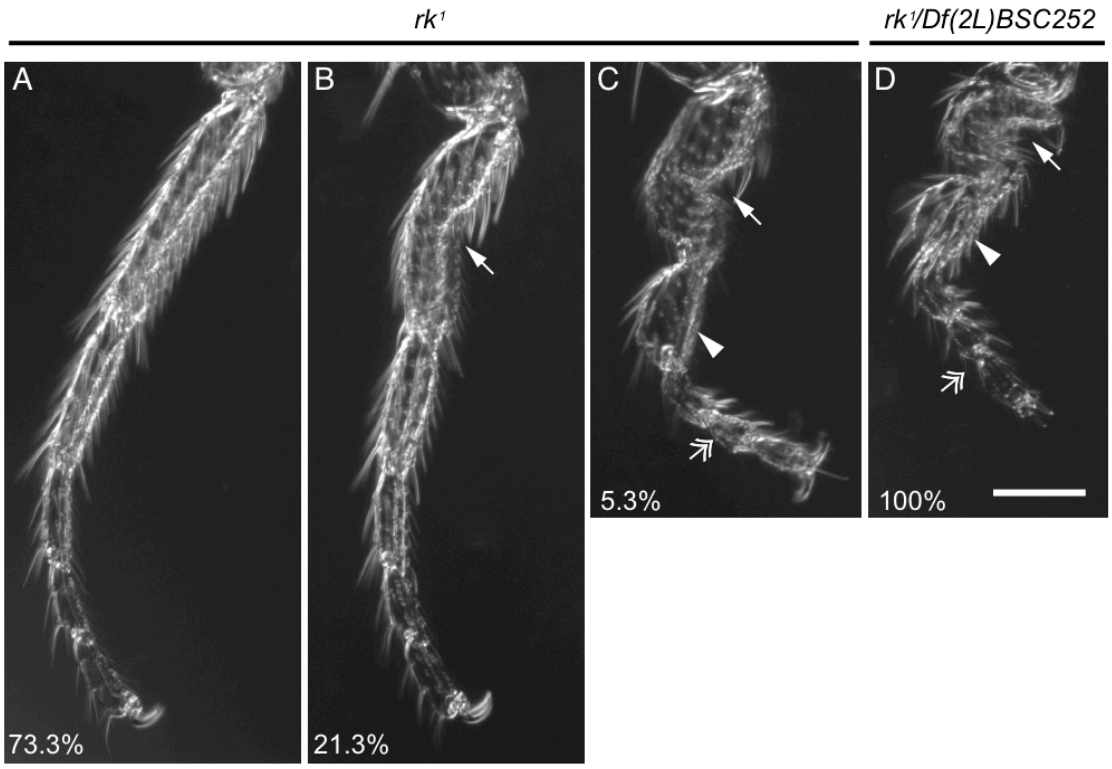
The homozygous rickets mutants *rk*^{1} and *rk*^{4} have previously been described as null mutants [2]. Consequently, gene silencing by ubiquitous RNAi expression should not result in a mutant phenotype that is greater than expected from a genetic 'loss of function' mutation. Surprisingly, the phenotypes documented in Figure 3.1 contradict this basic principle in two ways. First, the classic mutant alleles are perfectly viable as homozygous adults, as compared to Act5C(III)>*rk* RNAi (refer to Figure 3.1B). Second, although cuticle deformations are a hallmark of *rickets* mutations, neither mutant allele is crippled to the extent of Act5C(II)>*rk* RNAi adults (refer to Figure 3.1A). This unanticipated disparity in phenotypes suggests that either ubiquitous expression of UAS-*rk* RNAi has unintended consequences *in addition to* silencing of the *rickets* gene, or that *rk*^{1} and *rk*^{4} alleles do not truly represent genetic null mutants.

The *rickets* mutants were originally defined as nulls on the basis that the heteroallelic combination of two distinct chromosomal deletions which incompletely overlap with *rickets* is indistinguishable from homozygous *rk*^{1} and *rk*^{4} adults [2]. By necessity, our approach to assay whether *rk*^{1} and *rk*^{4} are loss of function mutants fundamentally differs from this earlier method, which used deficiencies that are no longer available.

To test if the *rk* alleles are homozygous nulls, we created hemizygotes by crossing either *rk*^{1} or *rk*^{4} to *Df(2L)BSC252/CyO*. Importantly, the *Df(2L)BSC252* deficiency contains molecularly defined breakpoints which delete *rk* in its entirety (including

surrounding genomic regions) [17]. In this scenario, hemizygous mutants, whether *rk*^{1}/Df or *rk*^{4}/Df, can only display a more severe mutant phenotype than homozygous *rk*^{1} and *rk*^{4} flies if these are not loss of function alleles. To test this hypothesis we had to select a suitable trait that might be enhanced when *rickets* is hemizygous with *Df(2L)BSC252*. For example, the complete penetrance of unextended wings in *rk*^{1} and *rk*^{4} makes this trait uninformative to score in a loss of function test. Indeed, the wings of both *rk*^{1}/Df and *rk*^{4}/Df flies are indistinguishable from the respective homozygous mutant alleles (data not shown). Another aspect of the *rickets* phenotype occurs with reduced penetrance: both *rk*^{1} and *rk*^{4} flies can exhibit a range of leg deformities including bowed femora and flattened tarsal segments [2]. We hypothesized that the reduced penetrance of leg deformities in homozygous mutants could be enhanced in hemizygous mutants. To determine the severity of *rickets* disruption, we scored deformities in metathoracic legs of both homozygous and hemizygous *rk*^{1} and *rk*^{4} flies. Remarkably, the penetrance of abnormal leg phenotypes in homozygous *rk*^{1} and *rk*^{4} stocks is increased when these alleles are hemizygous with *Df(2L)BSC252*. In a random sampling of homozygous *rk*^{1} legs (n=150) only 26.7% exhibit moderate or severely kinked tarsi, whereas 100% of *rk*^{1}/Df flies have severe tarsal deformities (Figure 3.2). A less drastic transformation was seen in our parallel *rk*^{4} study: whereas 100% of homozygous *rk*^{4} legs have tarsi with no observable defects (n=106), 66% of legs

Figure 3.2 Hemizygous legs are more kinked than homozygous *rk*^{1}. The homozygous *rk*^{1} stock displays incomplete penetrance of leg deformities. Leg deformities in *rk*^{1} homozygotes are classified as resembling wild-type legs (A), moderately kinked (B), or severely kinked (C). A moderate leg deformity is defined by the presence of a kink in the first tarsal segment (arrow). Severe leg deformities additionally exhibit a bulbous tarsal segment (arrowhead) and a rotated tarsal segment (feathered arrowhead). The corresponding proportion of leg deformities is given (A-C) for a random sample of adult metathoracic legs from the *rk*^{1} stock (n=150). (D) 100% of *rk*^{1}*/Df(2L)BSC252* animals exhibit the most severe leg deformities (n=64). This enhanced penetrance of indicates that the *rk*^{1} allele is not a null. The *Df(2L)BSC252* stock is balanced with *CyO*, and the legs of all *rk*^{1}/*CyO* resembled wild-type controls (data not shown). Scale bar = 0.1 mm.


rk¹
rk¹/Df(2L)BSC252
A
B
C
D
73.3%
21.3%
5.3%
100%

from *rk*^{4}/Df flies (n=42) were scored with tarsal deformities (data not shown).

A number of uneclosed *rk*^{1}/Df progeny were also observed. Could developmental lethality be affected in *rk*^{1} hemizygotes? We addressed this question by crossing *rk*^{1} to *Df(2L)BSC252/CyO, ActGFP*, to distinguish between heterozygous and hemizygous *rk*^{1} pupae. As expected, roughly equal numbers of *rk*^{1}/Df (n=53) and *rk*^{1}/*CyO, ActGFP* pupae (n=47) were obtained. However, only 60.4% (n=32) of *rk*^{1}/Df flies eclosed, as compared to 95.7% (n=45) of *rk*^{1}/*CyO, ActGFP*. In a parallel study, we compared developmental lethality in homozygous *rk*^{1} and heterozygous *rk*^{1}/+ flies. In this case, crossing the homozygous *rk*^{1} stock to *rk*^{1}/+ results in similar numbers of viable *rk*^{1} (n=38) and *rk*^{1}/+ (n=37) adult flies. These two experiments bolster our claim that *rk*^{1}/Df is indeed a stronger disruption of rickets function than homozygous *rk*^{1} by itself. The decreased eclosion rate in *rk*^{1}/Df appears to be due to an inability in otherwise healthy looking pharate adults to escape from the puparium.

We conclude that neither *rk*^{1} nor *rk*^{4} represent null alleles, when leg morphology and developmental lethality are taken into account. Therefore it is not unreasonable for Act5C>*rk* RNAi to display more severe phenotypes than the available homozygous *rk* mutant alleles.

rickets transcripts are found in several different tissues during development

As a prelude to manipulating *rickets* expression by RNAi with more selective GAL4 drivers, we asked where *rickets* might normally be expressed during development. A recent large-scale gene expression study had identified *rickets* transcripts in several tissues during development by microarray [22]. In their study, two larval tissues had quite abundant levels of *rk* transcripts: the CNS and the fat body. In addition to these tissues, we reasoned that since bursicon acts on the epidermis to tan the cuticle (see [6] for review), that *rk* may be expressed developmentally in epidermal tissue, or in tissue that eventually forms adult structures (i.e., the imaginal discs). We performed RT-PCR on tissue from 3rd instar larvae to search for *rk* transcripts (Figure 3.3). These results show that *rk* is indeed expressed in CNS (lane 1), fat body (lane 3), and body wall, containing epidermis (lane 4). We were unable to amplify *rickets* transcripts from our imaginal discs cDNA template (lane 2), most likely due to problems with concentrating enough tissue for the initial reverse transcription step. However, the presence of *rk* transcripts in larval wing imaginal discs has been identified in another large-scale microarray study [23]. Expression of *rickets* transcripts in the CNS is in agreement with bursicon's role in centrally mediating the wing expansion behavioral program (cf. [24]), but it is unclear what such a function, if any, could be in the larval CNS. It is currently unknown what function *rickets* expression serves in the fat body.

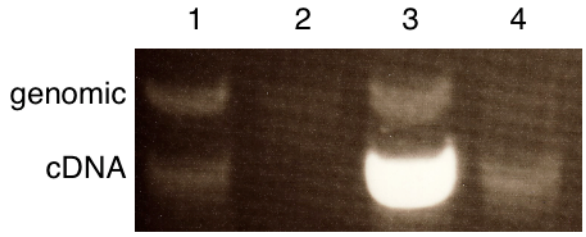


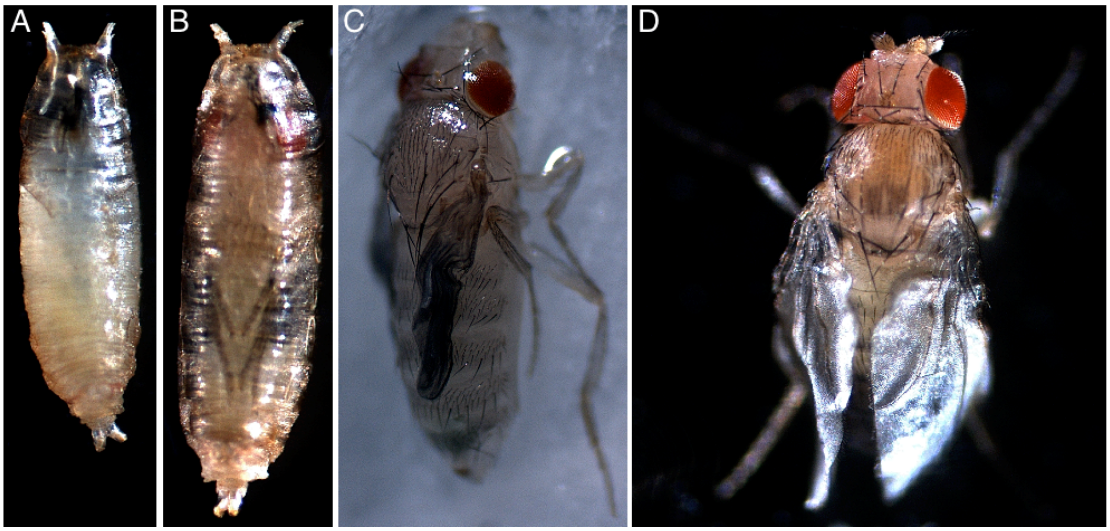
Figure 3.3 Transcripts of *rk* are developmentally expressed in different tissues from 3rd instar larvae. RT-PCR was used to analyze relative amounts of *rk* from w^{1118} 3rd instar larvae. Lane 1: CNS. Lane 2: imaginal discs. Lane 3: fat body. Lane 4: epidermis. Sizes on the gel correspond to the expected sizes for *rk* cDNA and genomic DNA, based on the primers used to amplify the cDNA template. Equal amounts of total RNA from each tissue were reverse transcribed. The intense cDNA band in Lane 3 is in agreement with the greater enrichment of *rk* in larval fat body as compared to other tissues (cf. [22]).

rickets expression is required in developing epidermal tissue and imaginal discs

We have just shown that *rk* transcripts are present in larval CNS, fat body, and epidermal tissue (Figure 3.3). These results suggest that targeted UAS-*rk* RNAi expression in separate tissues could reveal developmental requirements for *rickets* expression, as assayed by their mutant phenotypes. To test this hypothesis, we crossed UAS-*rk* RNAi to neural (*elav*, n-syb), peptidergic (CCAP, c929, 386, 36y) and fat body GAL4 lines (r^4, FB). With the above GAL4 drivers, expression of *rk* RNAi generated flies that appeared healthy and indistinguishable from wild-type flies (data not shown). We conclude that *rk* RNAi in CNS (or peptidergic neurons) alone is insufficient to generate *rickets*-like phenotypes.

In contrast, expression of UAS-*rk* RNAi by GAL4 lines with reported epidermal and/or imaginal disc expression patterns (cf. [25, 26]) produced several different mutant phenotypes. The observed *rk* RNAi phenotypes were dependent on the specific GAL4 driver. The resulting animals ranged from those unable to complete the prepupal stage, to healthy adult flies. For example, UAS-*rk* RNAi expression with T76-GAL4 results in 100% lethality prior to pupal ecdysis. From this genotype we only observed animals in small, misshapen and untanned pupal cases (puparia) (Figure 3.4A). Two separate drivers, T80-GAL4 and 69B-GAL4, give nearly identical results with UAS-*rk* RNAi. All T80>*rk* RNAi and most 69B>*rk* RNAi progeny die as pharate adults (Figure 3.4B). Apart from their failure to eclose, these progeny appear

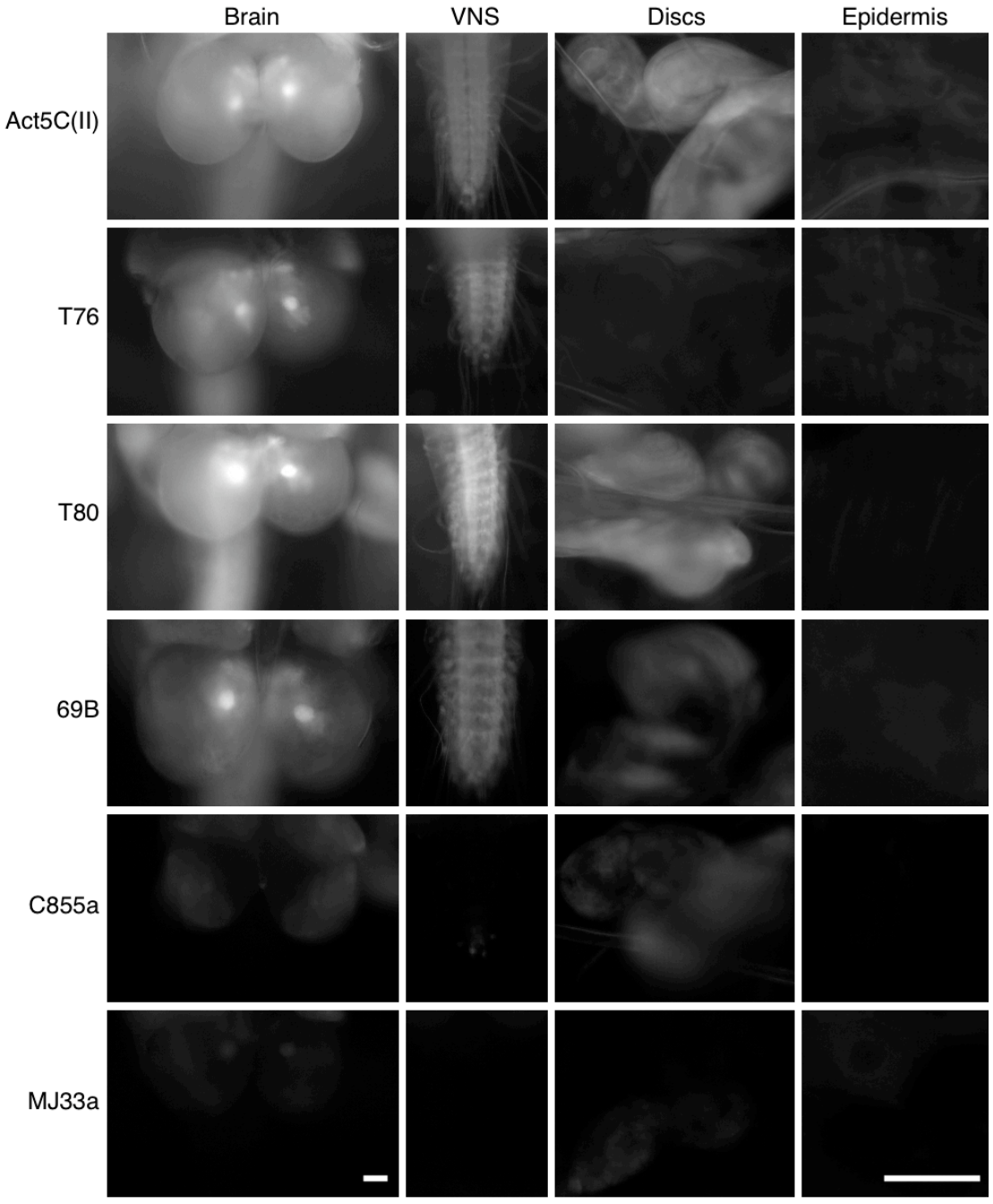
Figure 3.4 Phenotypes resulting from different GAL4 drivers of UAS-*rk* RNAi. (A) All T76>*rk* RNAi progeny die with small, soft, untanned puparia before pupal ecdysis. The puparia often have a slight crescent bend to them, rather than a straight orientation along the anterior-posterior axis. (B) In contrast, T80>*rk* RNAi develop into pharate adults, but all are then unable to eclose from their puparia and die. (C) A limited number of 69B>*rk* RNAi flies are able to eclose, but their legs are flaccid and collapse under the weight of the body. All flies die within 24 hours, before ever expanding their wings or tanning their cuticle. The majority of 69B>*rk* RNAi flies are arrested as pharate adults which cannot eclose (data not show), similar to T76>*rk* RNAi. (D) C855a>*rk* RNAi flies successfully eclose without any obvious problems. However, all adults have drooping, partially extended wings. This appears to be a result of the successful deployment of wing expansion behaviors in the absence of cuticular tanning. Metathoracic legs of C855a>*rk* RNAi adults resemble those of *rk*^{1}*/Df(2L)BSC252* (data not shown). A fifth genotype, MJ33a>*rk* RNAi, did not result in any observable mutant phenotypes (data not shown). Only A and B are shown at the same scale, to emphasize the smaller size of T76>*rk* RNAi pupae.


A
B
C
D

otherwise normal. Occasionally, adult 69B>*rk* RNAi "escapers" are observed (Figure 3.4C). Their untanned cuticle and unexpanded wings are reminiscent of *rk* mutants, yet severe cuticular defects in 69B>*rk* RNAi adults result in legs which cannot support their body weight. The absence of mobility in these flies likely contributes to their death within 24 hours after eclosion. Expression of *rk* RNAi with C855a-GAL4 does not effect the ability of progeny to eclose (Figure 3.4D). However, all C855a>*rk* RNAi progeny show great difficulty walking and climbing, as manifested by dragging their metathoracic legs during locomotion. A closer inspection of their leg morphology revealed kinked metathoracic tarsi which phenocopy the legs of rk^{1}/Df flies (data not shown). Unlike rk^{1}/Df flies, C855a>rk RNAi flies are able to expand their wings. Somewhat surprisingly, these wings never become rigid, resulting in a sagging appearance (Figure 3.4D). We also analyzed UAS-*rk* RNAi expression with MJ33a-GAL4; these flies are healthy and indistinguishable from wild-type controls (data not shown).

The variability in RNAi phenotypes that we observed is likely due to tissue-specific *rk* requirements. To determine where *rk* RNAi expression results in mutant phenotypes, we expressed UAS-*mCD8::GFP* and analyzed GAL4 lines for their expression pattern and relative levels in the larval CNS, imaginal discs, and epidermis. In the CNS (brain and ventral nervous system) there is either a strong solid pattern, as in Act5C(II), T76, T80, and 69B, or expression is severely restricted as in C855a and MJ33a (Figure 3.5). Interestingly, when the GAL4 lines with strong CNS

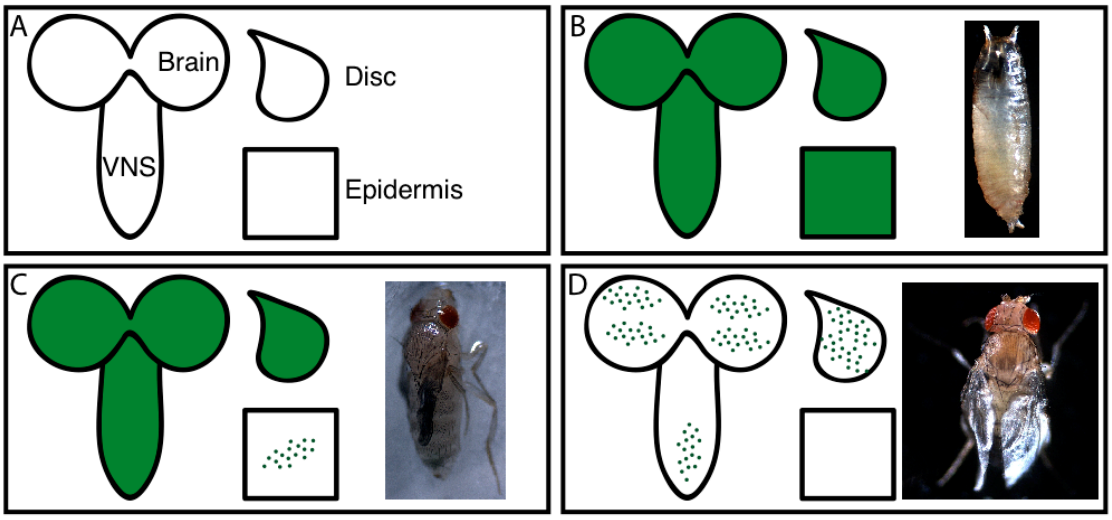
Figure 3.5 Expression patterns of GAL4 drivers in larval tissues. The membrane-bound GFP reporter UAS-*mCD8::GFP* was expressed with the drivers used in Figure 3.4 to assay for strength of expression and pattern in CNS, imaginal discs, and epidermal tissue. CNS is further divided into brain and ventral nervous system (VNS). As a point of reference for all other drivers, Act5C(II) expresses strongly in all tissue with solid patterns. T76 expresses strongly in CNS and epidermis. Expression in the imaginal discs is almost absent. T80 expresses strongly in CNS and imaginal discs, but is absent from epidermal tissue. 69B expression is strong in CNS and imaginal discs. It can also be detected in a very restricted pattern in the epidermal tissue. C855a expression is noticeably restricted in CNS and imaginal discs, as compared to Act5C(II). Expression is completely absent in the epidermis. MJ33a expression is restricted in the brain, but absent from the VNS, imaginal discs, and epidermis. For imaginal discs, examples from wing, haltere or metathoracic leg disc are shown. Epidermis refers to the epidermal tissue of the larval body wall.


Brain
VNS
Discs
Epidermis
Act5C(II)
T76
T80
69B
C855a
MJ33a

expression are used to drive *rk* RNAi the period of lethality varies considerably. This suggested to us to examine the effect of *rk* RNAi expression outside of the CNS, which would result in deformed prepupae (T76>*rk* RNAi), extraordinarily weak cuticle (69B>*rk* RNAi), or extended yet flaccid wings (C855a>*rk* RNAi).

The expression patterns of T76, 69B and C855a are summarized in Figure 3.6, along with their respective RNAi phenotypes. Among these drivers, T76 is unique in having strong solid expression in the larval epidermis (in addition to expression within CNS and imaginal discs). This suggests that *rk* RNAi expression in (but not limited to) the epidermis adversely affects puparium (and possibly pupal cuticle) formation (Figure 3.6B). The epidermal expression pattern of 69B is restricted to isolated patches, but there is strong solid expression in the imaginal discs (Figure 3.6C). Interestingly, abnormal leg shrugging is observed in 69B>*rk* RNAi animals which fail to eclose (data not shown), which could be related to the induction of extrication behaviors (see discussion). In combination with the flaccid leg phenotype in eclosed 69B>*rk* RNAi flies, these observations implicate a requirement for *rk* in strengthening the cuticle of legs before eclosion. All assayed tissue patterns are more restricted in C855a-GAL4 (Figure 3.6D). Imaginal discs and CNS show highly restricted patterns, but no expression is observed in the epidermis. These patterns imply that the limited *rk* RNAi expression in CNS and imaginal discs permits wing expansion without subsequent tanning.

Figure 3.6 Comparisons between GAL4 expression patterns and *rk* RNAi phenotypes. (A) Key to the schematics. Assayed tissues are labeled as brain, VNS, imaginal discs and epidermis. (B) Correspondence of T76 expression pattern with T76>*rk* RNAi phenotype. Solid expression in all tissues assayed appears to prevent the progression of prepupal development, resulting in small, untanned puparia. (C) Correspondence of 69B expression pattern with 69B>*rk* RNAi phenotype. Solid expression in brain, VNS, and imaginal discs, but weak pattern in epidermis, appears to result in flies with flaccid legs and unexpanded wings. (D) Correspondence of C855a expression pattern with C855a>*rk* RNAi phenotype. Extremely limited expression in all tissues assayed still results in adult flies whose wings cannot expand. The limited expression pattern likely allows *rk* expression required for wing expansion behaviors, but subsequent tanning of the wing is not possible, resulting in the sagging wing appearance. In the panels, solid green tissue symbolizes a solid GFP pattern observed in Figure 3.5, whereas the green punctae symbolize a restricted pattern of GFP expression.


A
Brain
VNS
Disc
Epidermis
B
C
D

Hormone release from CCAP neurons during metamorphosis coincides with rk RNAi phenotypes

The evidence in Figures 3.4 and 3.5 (summarized in Figure 3.6) suggests that the epidermis and imaginal disc-derived structures are responsive to bursicon hormone at different developmental stages. Since all T76>*rk* RNAi 3rd instar larvae appear normal yet die as prepupae, we reasoned that the deformed puparium phenotype must result from disrupted bursicon signaling at pupariation, or shortly thereafter. Functional evidence in support of a second wave of bursicon secretion during pupal development comes from 69B>*rk* RNAi, in which the majority of pupae die at the pharate adult stage. The legs of these pharate adults are generally so weak that they show abnormal shrugging and slipping movements, which could impede the initiation of ecdysis behaviors (see discussion). To investigate these corresponding developmental stages at which bursicon might be released, we expressed the GFP-tagged neuropeptide construct UAS-ANF-EMD with CCAP-GAL4. The UAS-ANF-EMD construct has been used in numerous studies as a reporter for secretion of endogenous peptide hormones during natively performed behavior [20, 27-29].

We observed ANF-EMD release from CCAP neurons in two localities at two different time points in live preparations. The first stage of release, from arborizations in the brain in the vicinity of the ring gland, occurred within 5 hours after puparium formation (APF)

and was complete by 7 hours APF (Figure 3.7). During these same early hours of prepupal development, ANF-EMD levels were unchanged at a possible secondary release site, the type III boutons at the NMJ (data not shown). A second phase of release is evident as ANF-EMD fluorescence levels at the NMJ decline just before pupal ecdysis, several hours after the first phase. This trend continued through pupal ecdysis until ANF-EMD fluorescence was almost absent from type III boutons, following leg elongation (Figure 3.8).

We believe that the prepupal ANF-EMD release documented in Figure 3.7 is consistent with the timing of the lethal phase in T76>*rk* RNAi (Figure 3.6B), suggesting that bursicon released at this stage is necessary to progress through prepupal development. Likewise, there is correspondence between the timing of ANF-EMD release from type III boutons after pupal ecdysis (Figure 3.8) and the arrested eclosion of 69B>*rk* RNAi due to flaccid legs (Figure 3.6C), indicating that bursicon is required to stiffen the legs of pharate adults.

3.5 Discussion

There is no question that the hormone bursicon, acting through its receptor rickets, is responsible for several post-eclosion events in *Drosophila*: deploying wing expansion behaviors [24], and plasticization and tanning of the new cuticle [1]. However, no cuticular tanning function earlier in development has been convincingly demonstrated. To our knowledge, ours is the first

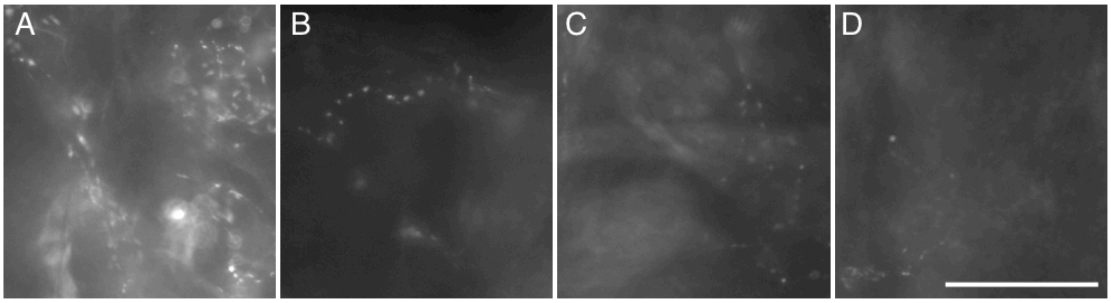
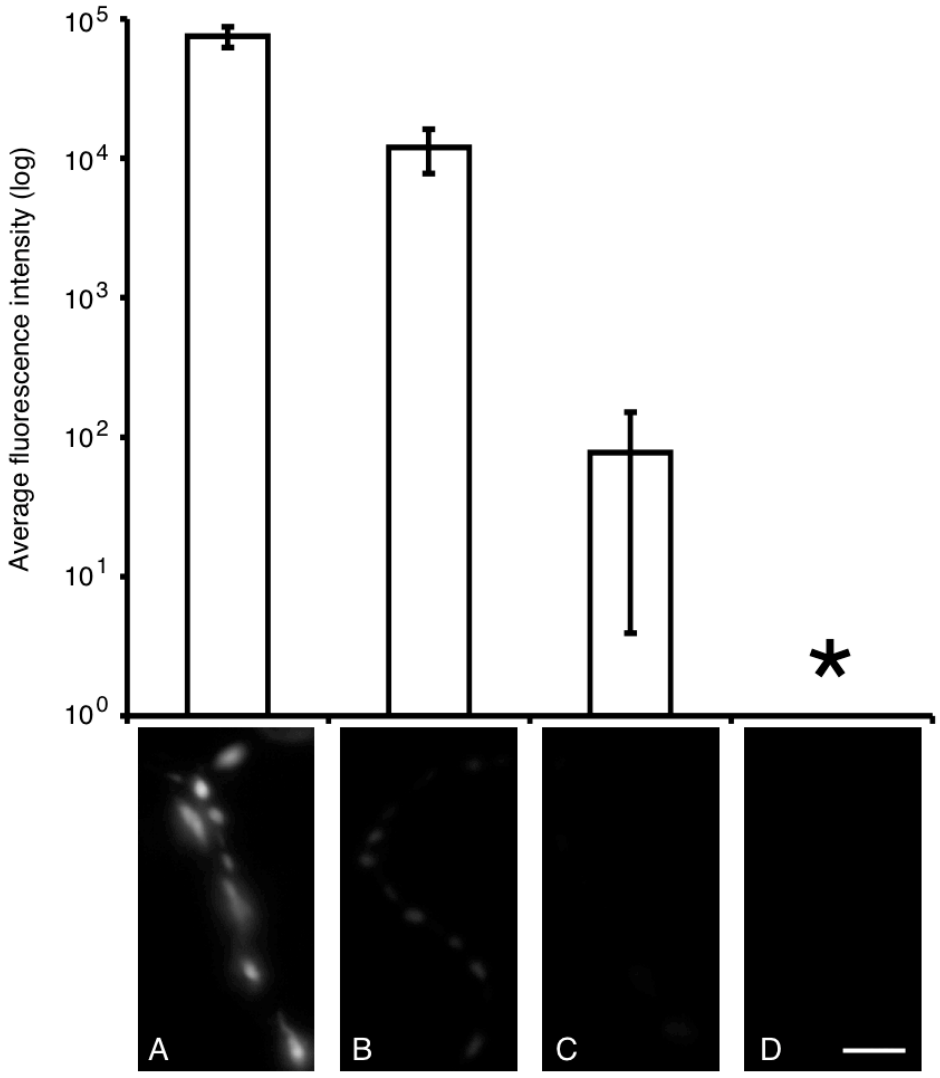


Figure 3.7 Timing of T76>*rk* RNAi developmental arrest coincides with hormone release from CCAP neurons (which also release bursicon). T76>*rk* RNAi larvae appear free from defects (data not shown) but arrest developmentally as prepupae (see Figure 3.4A). To examine the hormonal basis of this phenotype, the GFP-tagged neuropeptide reporter ANF-EMD was expressed in CCAP neurons. In the hours preceding pupal ecdysis, CCAP>ANF-EMD prepupae show a pattern of release in the arborizations of CCAP neurons within the brain. (A) 0 hours after puparium formation (APF). (B) 3 hours APF. (C) 5 hours APF. (D) 7 hours APF. These results indicate endogenous hormone release from CCAP neurons in the hours preceding pupal ecdysis. Scale bar = 50 μm.

Figure 3.8 Hormone release from type III boutons at the pupal NMJ coincides with pupal ecdysis. (A) Prepupa preceding pupal ecdysis by several hours. (B) Prepupa preceding pupal ecydsis by ½ hour. (C) Pupa immediately following head eversion, at pupal ecdysis. (D) Following elongation of leg discs, approximately ½ hour after pupal ecdysis. Average fluorescence intensity is reported on a log scale. For (D), no detectable fluorescence intensity is reported (indicated by the asterisk). These ANF-EMD release results are reliable indicators of endogenous hormone release from the CCAP neurons (which also release bursicon). We hypothesize that the absence of *rickets* expression in imaginal discs following this wave of hormone release is responsible for the flaccid leg phenotype observed in Figure 3.4C. Scale bar = 10 μm.


Average fluorescence intensity (log)
10^5
10^4
10^3
10^2
10^1
10^0
*
A
B
C
D

study in *Drosophila* to experimentally manipulate targets of bursicon to investigate the role of this “tanning hormone” prior to eclosion.

To do so, we relied on the use of a transgenic RNAi construct to disrupt *rickets* expression in separate tissue domains. Similarly, a recent study in *Drosophila* has effectively used targeted RNAi to reveal that expression of ‘sex peptide receptor’ is required in specific neurons of females to regulate their behavioral response to male seminal ‘sex peptide’ [30].

In retrospect our transgenic approach was also necessary to sufficiently silence *rk* expression, relative to available *rk* mutants. Our analysis of rk^{1} and rk^{4} provides clear genetic evidence that they are not null mutants. By focusing on leg deformities (an aspect of the *rk* phenotype which shows incomplete penetrance in homozygous stocks), we were able to show that 100% of both rk^{1} and rk^{4} hemizygotes are afflicted with leg deformities. These results underscore the utility of RNAi in gene disruption studies, even when genetic mutants are available.

The ability to enhance the penetrance of deformed leg segments when *rk* alleles are hemizygous with the *Df(2L)BSC252* deletion also suggests that some aspect of signal transduction is retained in *rk* mutants. Signal transduction in the rickets receptor activates PKA, through increasing cAMP levels [31]. Recently, post-eclosion tanning has been linked to phosphorylation of tyrosine hydroxylase (TH) by PKA, as a result of rickets activation [32]. A detectable TH activity profile in rk^{4} pupae and adults follows

the same pattern as TH activity in wild-type animals, yet at an attenuated level (cf. [32]). Although TH activity is an indirect measurement of rickets activation, we feel that the trend in TH activity shown by rk^4 pupae is in accordance with our view that *rk* alleles are not loss of function mutations.

By contrast, we currently do not know how the *rk* mutations could retain functionality, since they contain stop codons which should critically truncate the protein upstream of the transmembrane domain (rk^1) or within the transmembrane domain (rk^4) [2]. One possible scenario is that read-through of the stop codons is occurring at some level in these animals. The existence of this phenomenon has previously been demonstrated in *Drosophila*, in a rigorous analysis of read-through in the *Synapsin* gene [33]. To confirm a similar situation in *rk* mutants would require antibodies that recognize the rickets protein on either side of the stop codon. Regardless, a more thorough analysis of the function of rickets will have to await a mutation which exclusively removes its entire coding sequence.

The determination that *rk* mutants are not nulls validates our use of *rk* RNAi, which is lethal when expressed ubiquitously. Conveniently, the *rk* RNAi phenotypes we observed with more selective GAL4 lines (including imaginal disc or epidermal patterns) are consistent with a role for bursicon signaling at the epidermis to mediate cuticle tanning (cf. [32]). Even so, our results were still unexpected. T76>*rk* RNAi have a terminal phenotype with small, soft, misshapen puparia, apparently due to disrupted *rk* expression

in the epidermis. These prepupae strikingly resemble the prepupal lethal phenotype of various alleles of *Krüppel homolog* (*Kr-h*) (cf. [34]); *Kr-h* mutants are unable to respond to a critical prepupal peak of ecdysone [34]. T76>*rk* RNAi prepupae never advance to a point where a distinct pupa can be observed, which also suggests that *rk* expression in the epidermis may be important in formation of the developing pupal cuticle. The soft, flexible puparia of T76>*rk* RNAi is especially intriguing, since bursicon signaling has never before been directly linked to pupariation. Hardening of the puparium in Diptera is instead believed to be regulated by another peptide hormone, the "puparium tanning factor" [35-37], which has been identified as a pyrokinin in the grey flesh fly (*Sarcophaga bullata*) [37].

Two genotypes resulted in trapped pharate adults that were unable to eclose: T80>*rk* RNAi and 69B>*rk* RNAi. Fortuitously, we observed 69B>*rk* RNAi flies attempting to eclose, and a small fraction of these animals were successful. Based on the GAL4 expression patterns in imaginal discs of T80 and 69B, we hypothesize that *rk* is required to sclerotize the cuticle, and in particular the legs, during the pharate adult stage. The resulting legs of 69B>*rk* RNAi flies which successfully eclose are extremely flaccid and unable to support the weight of the fly (Figure 3.4C). Although leg movements have not been shown to be used for eclosion (cf. [38]), we believe that the failure to eclose in the majority of 69B>*rk* RNAi flies may be due to their flaccid legs. Mature 69B>*rk* RNAi pharate adults that failed to eclose exhibited

abnormal leg shifting movements within the puparium. These consisted of coxal shrugging movements, accompanied by displacement of the femora with slipping movements (data not shown). Previous results in the tobacco hornworm *Manduca sexta* [39] and also in *Sarcophaga bullata* [40] indicate that restraint of the legs are important stimuli to elicit extrication from the pupal case. Since the legs are defective in 69B>*rk* RNAi animals, the absence of this crucial leg restraint signal may lead to the observed failure at eclosion.

We also showed that a more restricted GAL4 pattern can result in less severe RNAi phenotypes. This is exemplified by C855a>*rk* RNAi flies, which succeed in expanding their wings following eclosion. Curiously the wings never harden, giving all C855a>*rk* RNAi wings a unique sagging appearance. Examination of the C855a expression pattern revealed limited CNS expression, especially in the VNS, whereas bursicon signaling within the CNS is required to deploy wing expansion behaviors [24]. Perhaps RNAi can be used in future studies to determine the CNS targets in this pathway with greater accuracy. To account for the “sagging wing” phenotype, the reduced expression pattern in C855a wing discs (relative to T80 and 69B) is evidently sufficient to disrupt tanning in the wings of C855a>*rk* RNAi. Finally, MJ33a>*rk* RNAi flies appeared normal, and from this we assume that their greatly restricted expression patterns in CNS, imaginal discs and epidermis are insufficient for disrupting *rk* expression.

Bursicon release is regulated by PKA activity within CCAP neurons [11], placing bursicon downstream of the other hormones involved in ecdysis behavior [1, 41, 42]. Intriguingly, hormonal release from CCAP neurons may not always follow after release of ecdysis triggering hormone, as the current model proposes (cf. [12, 21]). As we have shown in Chapter 2 of this thesis, the CCAP neurons release hormones from the type III boutons following the 2nd larval ecdysis, *but also* preceding initiation of the 2nd larval ecdysis. Similarly, we have observed two phases of release from CCAP neurons during pupal development: one of which occurs at 5-7 hours APF, and the second which coincides with pupal ecdysis (approximately 12-13 hours APF). We attempted to correlate the two lethal phenotypes obtained from T76>*rk* RNAi and 69B>*rk* RNAi with release of the exogenous ANF-EMD fluorescent neuropeptide reporter to strengthen our claims of spatial and temporal *rk* requirements. As it turns out, the lethal stages of T76>*rk* RNAi and 69B>*rk* RNAi do follow after phases of hormone release from CCAP neurons. Based on our ANF-EMD release study and the RNAi phenotypes, we hypothesize that bursicon release from CCAP arborizations is required during the early prepupal stage to tan the puparium. Curiously, there is no precedent for neuropeptide secretion of the hormones implicated in ecdysis at this early prepupal stage. We hypothesize that the second wave of bursicon release we observed is required to give rigidity to the cuticle (especially the legs) following pupal ecdysis. Nonetheless, our interpretations of these results are limited by the

fact that ANF-EMD release could represent the release of other hormones from CCAP neurons. A rigorous follow-up experiment to establish causation would be to disrupt bursicon hormone secretion separately in the CCAP arborizations and type III boutons, to see if the T76>*rk* RNAi and 69B>*rk* RNAi results can be replicated.

There are still some open questions regarding the ubiquitous expression of UAS-*rk* RNAi with the two different Act5C drivers. Curiously the Act5C(III)>*rk* RNAi phenotype is always more severe than Act5C(II)>*rk* RNAi, which could be an issue of driver strength. Considering that this is not an artifact, we were never able reproduce the larval lethal phenotype with more selective drivers, suggesting a developmental requirement for *rk* in multiple tissues.

It is also interesting that the larval lethal RNAi phenotype is not reproduced when bursicon-releasing CCAP neurons are transgenically ablated (or silenced). The most severe phenotype which occurs when CCAP neurons are ablated with UAS-*reaper* or electrically silenced with UAS-*Kir2.1* is that some proportion of progeny die at pupal ecdysis with incomplete head eversion [10, 11]. Surprisingly none of our RNAi phenotypes, including Act5C(II)>*rk* RNAi which survive to be pupae, phenocopy this head eversion defect. We can assume here that successful head eversion at pupal ecdysis is dependent upon a combination of additional hormones released from CCAP neurons at this stage. The disparity between RNAi and CCAP-ablated phenotypes suggests that either there is additional bursicon produced and

released outside of the CCAP-GAL4 pattern, or possibly that rickets receptor is activated by other ligands in addition to bursicon.

A final phenotype which we cannot account for in any of our RNAi experiments is the observation that both *rk* mutants and CCAP-ablated transgenic flies have characteristic short legs. While we were able to score many other leg defects, we never observed shortened legs in any of our RNAi experiments.

The peptide hormones which regulate ecdysis may interact in complicated ways which do not fit into a linear model of causation (cf. [43]). Here, we have shown that rickets activity, traditionally placed after eclosion, also has unexpected roles in tanning the puparium and strengthening pharate adult cuticle. These observations would not have been possible without silencing *rk* expression in tissues where it is required to respond to bursicon. It is noteworthy here to mention a recent study which examined the roles of all ecydsis-related peptide hormones and their receptors in the flour beetle *Tribolium castaneum*, using systemic RNAi [44]. Apart from effecting cuticular tanning, this study revealed that injections of *burs/pburs* or *rk* RNAi into pharate pupae resulted in diminished strength of contractions at pre-ecdysis. Our own study clearly indicates that rickets plays a central role prior to eclosion.

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CHAPTER 4

Summary

4.1 Brief overview

The results from this dissertation have already been discussed in Chapters 2 and 3. In this chapter I will summarize the key findings from each chapter, and propose a direction of research that can be taken from these results. Finally, I will also use this chapter to describe how my research interests developed during this project. This is an aspect of the scientific creative process that is overlooked in professional manuscripts, but which I feel may be appropriate in this venue, perchance to be of help to a future novice graduate student.

4.2 Summary of key findings for Chapter 2

The focus of Chapter 2 was to examine the role of the type III boutons at the larval NMJ. Two main questions drove the studies in this chapter: is the mechanism of release from peptidergic type III boutons similar to neurotransmitter release? And what is the functional significance of hormonal release from the type III boutons during larval development? The key findings from this chapter include:

1. Bursicon is present in the type III boutons at the larval NMJ. These peptideric terminals express the same secretory proteins that are believed to mediate release of classical

neurotransmitters: n-syb, CSP, Syx1A, SNAP-24, and SNAP-25.

2. Disruption of Syx1A expression in the CCAP neurons (some of which release bursicon) results in phenotypes strikingly similar to *burs* or *rk* mutants.
3. The type III boutons release a GFP-tagged peptide hormone, ANF-EMD, in two waves: just before ecdysis, and immediately following ecdysis. The ANF-EMD reporter is a suitable proxy for endogenous hormonal release. This indicates that a neuropeptide, possibly bursicon, is released just before ETH.

Prior to this study, the function of the type III boutons had been given little attention. To my knowledge, this is the first study to determine that the core synaptic proteins involved in neurotransmitter release are also expressed in these terminals. This is also the first study to identify a role for the type III boutons, by measuring hormonal release. These results are particularly striking, in that type III bouton secretion was observed prior to initiation of the ecdysis sequence. Current models of ecdysis initiation place the peripherally-released ETH upstream of hormones released from the type III boutons (which include CCAP and bursicon).

4.3 Summary of key findings for Chapter 3

The focus of chapter 3 was to identify the role of bursicon signaling during *Drosophila* development. The main question in

this study was: if bursicon promotes cuticle hardening following eclosion, does it perform a similar role after other ecdyses? The approach I took to answer this question was to focus on the targets of bursicon signaling, defined as tissues which express the bursicon receptor, rickets. The key findings from this study include:

1. The phenotypes of rk^{1} and rk^{4} are more severe when hemizygous with a *rickets*-spanning deficiency, as compared to homozygotes. Thus, contrary to previous descriptions, rk^{1} and rk^{4} are not genetic null mutants. It is more likely that these represent hypomorphs. This explains why ubiquitous expression of UAS-*rk* RNAi is lethal as early as the larval stages, whereas *rk* mutants are homozygous viable.
2. Developmental requirements for *rk* can be revealed by disrupting its expression in different tissues. This was achieved by narrowing down the expression of UAS-*rk* RNAi with selective GAL4 drivers. These results indicate that *rk* is required in the developing prepupa to tan the puparium. Expression of UAS-*rk* RNAi at this stage results in prepupae that neither tan their puparium, nor develop into pupae. These transgenic animals die at this stage.
3. Additionally, I showed that *rk* expression is necessary in the imaginal discs in order to impart sufficient rigidity to the cuticle prior to eclosion. The expression of UAS-*rk* RNAi in imaginal discs, particularly the legs, results in a very weakened cuticle. Strikingly, these weak legs may inhibit a

key proprioceptive signal which normally elicits eclosion behaviors.

4. Transgenic expression of the ANF-EMD reporter in CCAP neurons permits the tracking of hormone release from these neurons during pupal development. I found a phase of hormone release during the early hours of prepupal development which coincides with the prepupal lethal UAS-*rk* RNAi phenotype. I identified an additional phase of release from these neurons, after pupal ecdysis. This second phase may coincide with the requirement to tan the legs before eclosion, as observed in the lethal UAS-*rk* RNAi phenotype at eclosion. I hypothesize that these two phases of ANF-EMD release represent the secretion of bursicon, and that bursicon release at these stages contributes to the *rk* requirements I have outlined.

Prior to this study, bursicon and rickets had been demonstrated to be crucial to tanning of the new cuticle. However, strong genetic support for this role can only be shown in post-eclosion *Drosophila*. This is the first study of its kind in *Drosophila* to directly show that rickets is required at other developmental stages prior to adult eclosion.

4.4 Future directions

Two experiments would make a stronger case for the results in Chapter 2. First, to truly make the claim that Syx1A is required in the type III boutons to mediate release of bursicon, I would need

a way to specifically target the expression of UAS-Syx1A RNAi to the type III boutons. Instead, my CCAP>Syx1A RNAi experiment used the CCAP-GAL4 line which expresses in other CCAP-positive neurons in addition to the type III boutons. However, I am not presently aware of any GAL4 drivers which have a narrow expression pattern, restricted to the type III boutons. A second future experiment to improve the results in Chapter 2 would be to immunostain appropriately staged larval preparations with either CCAP or bursicon antibodies, to determine the identity of the hormones at the two phases of release in Figure 2.11.

The case for using UAS-*rk* RNAi to disrupt *rk* expression in Chapter 3 could be improved by including an RT-PCR experiment which analyzes relative levels of *rk* in Act5C>*rk* RNAi animals, compared to controls (including wild type, Act5C-GAL4, and UAS-*rk* RNAi animals). The hypothesis is that *rk* transcripts would be (nearly) absent in Act5C>*rk* RNAi, relative to the controls, and thus this experiment would be used to confirm that the UAS-*rk* RNAi construct does indeed knock-down *rickets* transcript levels. An interesting future direction for the experiments in Chapter 3 would be to address the role of *rk* in the fat body. It is truly surprising how much *rk* is expressed in the larval fat body relative to other larval organs, which may explain why I was unable to obtain any mutant *rk* RNAi phenotypes with 3 different fat body GAL4 drivers – it is possible that UAS-*rk* RNAi expression in 'wild type' fat body is not sufficient to silence *rk* expression. Perhaps a better approach would be to express UAS-*rk* RNAi in the fat body in a sensitized

background, where *rk* gene expression is already reduced – such as in a mutant background (rk^1 or rk^4) or with a deficiency that deletes the *rickets* gene.

4.5 Research development sketch

The ideas behind Chapter 2 began with a project which has never seen the light of day. The initial idea for this project was to study the expression of exocytotic proteins in the peripheral Inka cells. These cells produce and release ETH, which triggers ecdysis behaviors. However for various histological reasons, the Inka cells were not amenable to convincing immunoreactive staining, except for ETH – which is not very informative! At this point I turned my interest to the type III boutons. Surprisingly, even though a variety of peptide hormones have been identified in these boutons, no studies had examined the functional significance of the release sites at the larval NMJ. This was the initial inspiration for the experiments in Chapter 2.

The experiments in Chapter 3 were for me a logical extension of the results in Chapter 2. If the type III boutons release hormones, possibly including bursicon, during larval development, what is the role of bursicon signaling during development? Although bursicon signaling, through its receptor rickets, has been shown to mediate cuticle tanning, this has only been demonstrated genetically in *Drosophila* following adult eclosion. For me, this begged the question: do bursicon and rickets perform the same function after all ecdyses? Previously published results, in which

the CCAP neurons are transgenically ablated with UAS-*rpr* or electrically silenced with UAS-*Kir2.1*, provided some additional inspiration for these experiments. These ablation experiments result in head eversion defects at pupal ecdysis, which could be due to defective release of CCAP, bursicon, or a combination of the two. My interest in examining developmental roles for bursicon was also stimulated by some unpublished experiments which suggested that deletion of the CCAP gene had no effect on any ecdysis throughout development. These combined observations suggested to me that bursicon was much more important during *Drosophila* development than indicated by the phenotypes of *burs* or *rk* genetic mutants.